INTRODUCTION

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Availability of nitrogen fertilizer is the most important factor that limits plant productivity. To increase crop yield application of industrially synthesized nitrogen fertilizer has increased by a factor of 10 over the last 25 years. Currently, nearly 55 x 10^6 tonnes of nitrogen fertilizer is consumed every year throughout the world. However, most of this fertilizer is used only in developed countries while 77% of the world population is shared by the developing countries. To meet the demand of food in the developing countries application of increased nitrogen fertilizer is necessary but is restricted due to economic reasons. Besides, use of synthetic fertilizer also adds to the environmental pollution. As such exploitation of biological nitrogen fixation is gaining increasing appreciation throughout the world.

Although about 77% of atmospheric gases consists of nitrogen, higher organisms including plants and animals cannot use it directly. Only certain microbes are endowed with the genetic capability of catalyzing reduction of atmospheric nitrogen to ammonia ; these diazotrophs are distributed over many genera and can be classified under three general categories ; symbiotic, associative and free-living. Symbiotic association with higher

plants appeared to have evolved only three types of diazotrophs that benefit plants through dinitrogen fixation. These are (a) species of Rhizobium and Bradyrhizobium that form root-nodules in leguminous plants, (b) Frankia, an actinomycete like soil microbe that infects and develops nodules in roots of Alnus, Comptonia, Casuarina, and a few other trees, and (c) heterocystous cyanobacteria, which may live in symbiotic association with a variety of plants.

Rhizobium-legume symbiosis has the highest potential of nitrogen fixation and it was estimated that this form of symbiosis contributes almost 40 x 10^6 tonnes of nitrogen annually to grain legumes (Hardy & Havelka, 1975). On a global basis root-nodule symbiosis contributes almost 80% of the total biologically fixed N_2.

Taxonomically, the root-nodule bacteria that enter into symbiotic relationship with leguminous plants were grouped under the genus Rhizobium in the eighth edition of Bergey's Mannual of Determinative Bacteriology (Jordan & Allen, 1974). Recently, on the basis of their physiological (Graham, 1964 ; Norris, 1965 ; 't Mannetje, 1967 ; Moffett & Colwell, 1968), biochemical (Martinez de-Drets & Arias, 1972 ; Skotnicki & Rolfe, 1977) and genetic (Herberlein et al. 1967 ; Gibbins & Gregory, 1972 ; De Smedt & De Ley, 1977 ; Crow et al. 1981) characters these have been separated under two generic heads, Rhizobium and

Bradyrhizobium (Jordan, 1984). The classification of family Rhizobiaceae as revised is shown in Table 1. The members of the genus Rhizobium are fast-growing, having mean generation time of 2-4 hours while members of the genus Bradyrhizobium are slow-growing ones having mean generation time of about 6-8 hours in yeast extract-mannitol medium (Vincent, 1970).

Root-nodule bacteria are capable of entering into symbiotic association with more than 1130 species of leguminous plants and Parasponia among Ulmaceae (Trinick, 1973 ; Vincent, 1974). The bacteria incite production of nodules on the roots of these plants by a complex developmental process. Within the nodules the bacteria enlarge and change shape into bacteroids and fix nitrogen within the protected niche of root-nodules at the expense of plant photosynthates. While photosynthates are allocated to various sink organs of plants, nodule represents one of the strongest sinks, receiving an estimated 30% or more of the net photosynthates (Rawsthorne et al., 1980). Photosynthates supplied to nodules are used for (a) generation of bacterial reducing power and supply of ATP to the nitrogenase system, (b) maintenance of normal host-cell cytosol metabolism, and (c) a supply of carbon skeletons, ATP and reducing power for the synthesis of nitrogenous compounds which are then exported selectively back to the growing vegetative and reproductive structures of the host plant via xylem (Pate et al., 1979, 1981). A schematic diagram of the events that take place inside a root-nodule is presented (Fig.1). Burns and Hardy, (1975) ;

Zumft and Mortenson, (1975) and Winter and Burris, (1976) separately reviewed that biological N_2 fixation requires a large input of metabolic energy. Evidence supported that supply of energy may often be a rate limiting step in symbiotic N_2 fixation (Hardy & Havelka, 1975). The mechanisms for the production of large amounts of energy required for the reduction of atmospheric nitrogen are manifested in the carbon catabolic sequences. Shanmugam et al., (1978) commented that a complete picture of nitrogenase regulation would have to include control of ATP and reductants, essential substrates for the nitrogenase reaction.

It appears that the major corollary to symbiotic N_2 fixation is the supply of NH_4^+ to the host. NH_4^+, the first stable product of N_2 fixation, is excreted by Rhizobium bacteroids (Bergersen & Goodchild, 1973) into the host cell cytoplasm where it is assimilated and used in the synthesis of organic N for transport. N_2 fixing plants can be classified as amide exporters or uride exporters based on the composition of the xylem sap collected from the excised nodules or nodulated root-systems. The amide exporters transport asparagine, glutamine or 4-methylene glutamine while uride exporters export either allantoin and allantoic acid or citrulline. Legumes of temperate origin including pea, broad bean, alfalfa, clover and peanut are reportedly amide exporters whereas, tropical legumes of the tribe phaseoleae, soybeans and cowpeas

synthesize and transport urides from recently fixed N_2.

Several laboratories (Burley, 1961 ; Pate, 1962, 1975) identified sucrose as the major photosynthetic product which is transported to the nodules for further metabolism. Lawrie and Wheeler, (1975) indicated that although sucrose is the major carbon supplied to the plant root, it was not detected in nodules in sufficient amounts. Interestingly, however, Antoniw and Sprent (1978) found sucrose to be more intensely labelled than glucose in Phaseolus nodules. A positive correlation between the concentration of sucrose fed to excised nodulated cowpea roots and N_2 fixing activity indicated that sucrose or a product of its metabolism may be the primary carbon source for the nodules (Yashida & Yatazawa, 1977). On the contrary, sucrose does not stimulate N_2 fixation in isolated soybean bacteroids (Bergersen & Turner, 1967). Kidby (1966) observed neutral invertase- an enzyme responsible for sucrose break down to be present in nodules but absent in isolated bacteroids which led him to believe that sucrose plays a vital role in N_2 fixation. Thus, the precise details of carbon sources involved in stimulation of N_2 fixation is confusing and it is also not quite known how they are utilized to support nitrogen fixation in different legumes.

Using sugar non-utilizing mutants of R. leguminosarum in nodulation Glenn et al., (1984) concluded that the capacity to

utilize C-6 and C-12 sugars is apparently not essential for bacteroid development or establishment of effective N_2 fixation. On the otherhand, use of dicarboxylic acid transport (DCT) mutants revealed that a supply of C-4 dicarboxylic acids is essential for normal nodule function in R. leguminosarum var trifolii and R. leguminosarum var viceae (Ronson et al.,1981 ; Finan et al., 1983 ; Arwas et al., 1985). Mutants of these organisms defective in dicarboxylate transport (dct^- mutants) form nodules and differentiate into bacteroids (Nod^+) but do not fix nitrogen (Fix^-). Kurz and La Rue, (1975) and Pagan et al., (1975) separately demonstrated in vitro nitrogenase activity of some slow-growing strains with succinate and pentose sugar supplemented to the growth medium. Later, Pankhrust (1981) also observed that succinate was required to support ex planta N_2 fixation. These observations provided evidence that dicarboxylates are essential substrates required for N_2 fixation. However, the regulation leading to the availability of dicarboxylates for the bacteroids during N_2 fixation has yet to be fully understood. The understanding of the production of large amount of energy needed as the activation energy for the reduction of nitrogen from catabolism of organic molecules is fundamental to improvement of N_2 fixation capacity of symbiotic system. From an understanding of carbon catabolism, it is also expected, specific regulatory mechanisms of metabolic pathways involved in the production of energy will be identified. However, knowledge as to the biochemical routes for metabolism of C-4 dicarboxylates

for the production of ATP during N_2 fixation is still in its infant stage.

The metabolism of other readily available carbon sources, such as alcohols, in nodules although studied (Peterson & La Rue, 1981) but is still not understood in sufficient detail and further studies are needed to reach a consensus of the impacts of dicarboxylates over the metabolic sequences of these compounds. This necessiates the identification of the regulatory features of N_2 fixation in presence of sucrose, glucose or other nodule carbons in bacteria or in any of their mutant forms.

Studies on carbon nutrition of free-living rhizobia revealed that fast-growing rhizobia are capable of growing on a variety of carbon sources, whereas slow-growing bradyrhizobia cannot use di- and tri-saccharides and certain organic acids of the TCA cycle. On the other hand, slow-growing bradyrhizobia are capable of growth on aromatic carbon compounds (Parke & Ornston, 1984). Previous investigations have shown that all rhizobia irrespective of their growth properties possess the Entner-Doudoroff (ED) pathway and the tricarboxylic acid (TCA) cycle for glucose oxidation (Katznelson & Zagallo, 1957 ; Keele et al., 1969, 1970 ; Martinez de-Drets & Arias, 1972 ; Ronson & Primrose, 1979a). Using cells cultured on glucose, the occurrence of pentose phosphate (PP) pathway was demonstrated in strains of fast-growing Rhizobium which posses NADP linked 6-phosphogluconate dehydrogenase (6-PGD), the key enzyme for the

pathway. Lack of the enzyme 6-PGD in glucose grown slow-growing Bradyrhizobium laid the foundation of the enzymatic basis of differentiation of Rhizobium and Bradyrhizobium (Martinez de Drets & Arias, 1972). Evidence for the presence of Embden Meyerhoff Parnas (EMP) pathway, however, is contradictory. There are a few reports (Katznelson & Zagallo, 1957 ; Martinez de Drets & Arias, 1972 ; Siddique & Banerjee, 1975 ; Mulongoy & Elkan, 1977 ; Stowers & Elkan, 1983) of the presence of significant level of glycolytic enzymes, phosphofructokinase (PFK) and fructose-1,6-biphosphate aldolase in these organisms, while others failed to detect them. For gluconate catabolism rhizobia follow ED-pathway and possibly an ancillary ketogluconate (KG) pathway (Keele et al., 1970).

Most of the natural environments are scarce in nutrients including readily metabolizable carbon sources. Rhizobial inoculum during the period outside the host plant faces a femine condition when the bacteria are starved of various nutrients. It is possible that during such a period the regulation of carbohydrate metabolic system is modulated and becomes coupled to available metabolic reserves for greater survivial. Till date, no attempt has been made to unveil the metabolic pathways and their regulatory mechanisms in Rhizobium during adaptation to starved situations. It is imperative that such studies are undertaken with an objective of increasing the survivality of Rhizobium inoculum as well as their nodulation and N_2 fixation

efficiencies.

Although rhizobia have the capability of metabolizing sugars and organic acids at their disposal during free-living state or as bacteroids in legume nodules, the availability of such carbon sources is severely limited in soil and survival of rhizobia in soil may be dependent upon the utilization of aromatic compounds which are available in soil from complex plant polymers such as lignin.

During carbon femine, as soil environment often provides, conservation of carbon for biosynthetic events is very essential. Under such condition operation of an anapleurotic glyxylate pathway for gluconeogenesis and carbon conservation (Lamb et al., 1978 ; López-Boado et al., 1987 ; Thomas & Baxter, 1987) has been reported.

The work embodied in this dissertation attempts to understand the basic biochemical mechanisms involved in carbon catabolism, specific metabolic pathways and their regulations under conditions of provision of different carbon sources as well as during carbon nutrition femine in Rhizobium of Cicer arietinum L.

The choice of Rhizobium species (Cicer arietinum) was dictated primarily by the fact that chickpea is the third most widely grown legume in the world and is grown extensively in

many regions of India and occupies the position of a major agricultural legume (Medhane & Patil, 1974) with an annual production of 4919 thousand tonnes during the year 1985-1987 in an area of 7163 thousand ha. Rhizobia infecting Cicer were thought to warrent the formation of new separate species based on the studies of serology and intrinsic antibiotic resistance (Kingslay & Bohlool, 1983), host specificity (Gaur & Sen, 1979), indigenous plasmid analysis (Cadshia et al. & Ruiz Argiles, 1986) and by DNA-hybridization (Chakrabarti et al., 1986). It was hoped that studies on the carbon metabolism and its regulation in free-living root-nodule bacteria of Cicer arietinum would provide a baseline from which developmental events in the dissimilation of carbon in its bacteroids may be recognized.