1 INTRODUCTION

Azolla is a genus of aquatic ferns, floating on the water surface normally containing a symbiotic, heterocystous cyanobacterium, *Anabaena azollae*, within its cavities formed in their aerial dorsal leaf lobes. The symbiotic *Anabaena* filaments can provide the association with its total N requirement by the fixation of atmospheric dinitrogen. The reported rate of N_2 fixation ranged from 1.0 to 3.6 kg N ha^{-1} day^{-1} (Watanabe, 1982). *Azolla* is well documented for being used as fertilizer in the rice paddies of southeastern Asia, especially China and Vietnam for centuries. It receives much attention from agriculturists and botanists because it is the world's most important economic fern.

Rice accounts for 21% of the total energy content of the world's food. Since rice provides more calories per hectare than any other cereal crop, it is important as a food crop. The success of rice production in tropic and subtropics depends on an efficient and economic supply of N, a nutrient often limiting to plant growth.

The application of *Azolla* as a bio-fertilizer on agricultural crops production, in order to provide with the crucial nutrient nitrogen as a natural source and to reduce the chemical fertilizers, can be beneficial to the future of sustainable land use. But, one of the most important factors limiting the *Azolla* utilization is the expenditure of labor. Despite the cultivation problems, it would do well to depend on *Azolla* as a fertilizer for rice that is as environmentally safe and energy efficient.

Azolla has several other uses besides fertilizing rice. It can be fed to cattle, hogs, ducks, chickens, and carp. It can serve as compost because it decomposes rapidly in some case as little as seven to ten days. It can even be cultivated as an ornamental in ponds or aquatic gardens because it turns brilliant red in the fall. It may also be used for hydrogen fuel production, biogas production, the control of weeds, the control of mosquitoes, and the reduction of ammonia volatilization that accompanies the application of chemical N fertilizer. The present of *Azolla* cover on the surface of flooded water has been proved to significantly reduce volatilization of applied N-fertilizer (Vlek et al., 1992; Vlek et al., 1995). These observations have been confirmed in paddy fields (Kumarasinghe and Eskew, 1993; Macale, 2002). The reasons for this effect have not been completely concluded. However, it is clear that the effect of *Azolla* depressed the algae photosynthesis in the underlining water. In part, another reason might be due to the direct assimilation by *Azolla* of urea-derived ammonium (Cisse, 2001). The effect of an *Azolla* cover on the floodwater pH is partially explained in terms of its absorption of available light (Vlek et al., 2002).

Under optimized conditions *Azolla* can double their biomass in less than two days with dinitrogen as the only N source and accumulate 5-6.5% N based on a dry weight (Peters et al., 1980). *Azolla*-*Anabaena* symbiosis is noted for its high productivity combined with its ability to fix dinitrogen at high rates. Consequently *Azolla* has been used for centuries to fertilize rice paddy avoiding the need for manure. The interactions in the nitrogen metabolism between the partners of this symbiosis have been the subject of a number of investigations. However, the extent of interaction in the carbon metabolism between the symbiotic partners has received relatively little attention.

Both partners of the association are photosynthetically competent and their pigments are complementary. *Anabaena* performing photosynthesis in its vegetative cells should be able to grow and to provide its host with N without being dependent on C-transfer. However, there are clear indications that the cyanobiont contributes only little to the total carbon fixation of the *Azolla*-*Anabaena* symbiosis and that carbon required by the endophyte to a large part is provided by the host (Kaplan and Peters, 1988). However, ATP necessary to drive nitrogen fixation seems to be produced photosynthetically in the *Anabaena*-heterocysts. The present thesis is intended to contribute informations concerning the carbon budget of the *Azolla*-*Anabaena* association, above all the relation between photosynthetic C-yield in light and respiratory C-loss in dark and the participation of the endophytic *Anabaena* population in carbon photosynthetically assimilated by the association.

The species most studied is *Azolla caroliniana*. However, the *Azolla* widely distributed in Asia and more important for rice production are *Azolla pinnata ssp. imbricata*. As is stated in more detail in the next chapter the two species are grouped into the two different subgenera of genus *Azolla*. Therefore it was originally intended to compare both species. It turned out, however, that some aspects were difficult to handle with *Azolla pinnata ssp. imbricata*.

2 REVIEW OF LITERATURE

2.1 Prominent features of the *Azolla-Anabaena* symbiosis

The *Azolla* sporophyte consists of branched, floating stems bearing deeply bi-lobed, alternately arranged leaves and adventitious roots. Chloroplasts exist in the unicellular root hairs and in the cortical layers of the root. Transfer cells have been detected in the roots (Duckett et al., 1975). *Anabaena* cyanobionts together with bacterial endophytes are enclosed in cavities formed at the bases of the aerial dorsal leaf lobes. The cavities communicate with the outside by a pore.

A colony of undifferentiated, or generative, *Anabaena* filaments are associated with the apical meristem of each stem. The meristem and *Anabaena* filaments are enclosed and protected by the developing bi-lobed leaves. During stem growth, some filaments are partitioned from the *Anabaena* colony into the newly forming cavities. The interior surface of each leaf cavity is lined with an envelope (Peters, 1976), which is filled with mucilaginous material embedding the endophyte and multicellular hairs from the cavity epidermis. The center of a mature cavity is probably occupied by a liquid or gas bubble (Uheda et al., 1995; Caiola and Forni, 1999), but free from mucilage, cyanobacteria or bacteria. The mucilage is rich in amino acids, ammonium compounds (Canini et al., 1990; Kitoh et al., 1992) and carbohydrates. There is still controversy about the origin of mucilage polysaccharides (Peters et al., 1978), formation by *Anabaena* (Robins et al., 1986) or, at least partly, by the accompanying bacteria (Forni et al., 1992). However, Forni et al. (1998) reported evidence that all three partners of the symbiosis are involved in the production of mucilage polysaccharides inside the *Azolla* leaf cavity.

An ontogenetic sequence of the host and endophyte is expressed along each axis of the floating stems (Hill, 1975 and 1977; Calvert and Peters, 1981). The generative *Anabaena* filaments at the stem apex perform complete photosynthesis but do not have heterocysts and nitrogenase. With ongoing maturation and growing distance from the frond tip the photosynthetic activity of vegetative cells is reduced to very low rates and heterocysts are differentiated instead (Rai et al., 2000).

Envelopes containing the endophytes and multicellular hairs embedded in the mucilage can be isolated from leaf cavities by digesting *Azolla* leaves with a mixture of cellulolytic enzymes (Uheda, 1986a). In spite of the cyanobacterial nature of *Anabaena* those entities often are designated as algal packets.

Anatomical studies have shown that two types of trichomes, namely simple and branched hairs, become intimately associated with *Anabaena* filaments in leaf cavities (Calvert and Peters, 1981; Peters and Calvert, 1983). Each cavity has only 2 primary branched hairs. They occupy a well-defined position in the cavity in that they are located on the path of the foliar trace (Duckett et al., 1975). As development and maturation proceed, a second branched hair and a population of up to 25 simple hairs is formed in each cavity. Simple hairs are randomly distributed around those portions of the cavity bordered by mesophyll (Calvert and Peters, 1981). The branched hairs may participate in exchange of fixed nitrogen between the symbiotic partners and simple hairs may be involved in exchange of carbon compounds from *Azolla* to *Anabaena* (Calvert et al., 1985).

An unique feature of the relationship between *Azolla* and *Anabaena azollae* is the one of permanent symbiosis: the two organisms are associated in all stages of the life cycle of the fern and the association persists from one generation to the next regardless of whether reproduction is sexual or asexual (van Hove, 1989). No other cyanobacterial species occurs as a symbiont of *Azolla* ferns. However, a small population of non-nitrogen fixing bacteria (Peters, 1977) does exist, such as *Pseudomonas* (Bottomley, 1920; Shi and Hall, 1988) and *Arthrobacter* (Wallace and Gates, 1986; Carrapico, 1991; Nierzwicki-Bauer and Aulfinger, 1991). A few bacteria are always present both in the cavities of symbiotic association and *Anabaena*-free *Azolla* (Peters, 1976). The role or function of the bacteria is not yet clear though a mutual dependence of *Anabaena* and bacteria has been assumed (Lobakova et al., 2001).

The symbiosis between *Azolla* and *Anabaena* has been said to be obligate in the sense that no one of the partners can be grown isolated (Rai et al., 2000). In fact, *Azolla* invariably harbours the cyanobiont in nature. However, *Anabaena*-free or asymbiotic *Azolla*, though growing very slowly, can be obtained by some methods (see 2.5). Several authors have claimed to have grown *Anabaena azollae* separated from its host (Newton and Herman, 1979; Gebhardt and Nierzwicki-Bauer, 1991).

The species in the genus *Azolla* have been grouped into two sections (subgenera): *Euazolla* and *Rhizosperma*, based on the type and number of their megaspore floats. The section *Euazolla* includes *A. caroliniana* Willdenow, *A. filiculoides* Lamarck, *A. mexicana* Persl, *A. microphylla* Kaulfuss and *A. rubra* Brown. The section *Rhizosperma* includes *A. nilotica* Decasine and *A. pinnata* Brown with two subspecies, *A. pinnata pinnata* and *A. pinnata imbricata* (Lumpkin and Plucknett,1980).

Azolla occurs in freshwater habitats in tropical, subtropical, and warm temperate regions throughout the world. Prior to intervention by man, *Azolla caroliniana* was distributed in eastern North America and the Caribbean, and *A. pinnata* in most of Asia and the coast of tropical Africa (van Hove, 1989).

2.2 Endophyte *Anabaena*

The filamentous nitrogen fixing cyanobacterium associated with all *Azolla* species was earlier referred to as a single species, *Anabaena azollae* Strasburger. It may, however, be more related to the genus *Nostoc* rather than to *Anabaena* (Meeks et al., 1988; Peters and Meeks, 1989; Plazinski et al., 1990). *Anabaena azollae* strains associated with different *Azolla* species are genotypically not identical (Lejeune et al., 1999). All cyanobionts from the seven *Azolla* species can be distinguished by molecular genetic methods such as polymerase chain reaction (PCR) fingerprinting (Zheng et al., 1999). This led to suppositions that the groupings of cyanobionts are more or less parallel their corresponding host plant in the revised taxonomy tree (van Coppenolle et al., 1993) and supports the hypothesis of co-evolution between *Azolla* and its cyanobionts (Caudales et al., 1995). Results from restriction fragment length polymorphism (RFLP) analyses suggested the simultaneous presence of different cyanobacterial symbionts in *Azolla* spp. (Gebhardt and Nierzwicki-Bauer, 1991).

The endophyte *Anabaena azollae* consists of unbranched trichomes containing bead-like or barrel-formed, heavily pigmented vegetative cells, approximately 6 σm in diameter and 10 σm in length (van Hove, 1989), and intercalary heterocysts which are slightly larger and more pale in colour having thicker cell walls. In free-living *Anabaena* or *Nostoc* the distance between two heterocysts is 15-30 vegetative cells, i.e. a frequency of 3-6%, depending of the concentration of available nitrogen compounds. A remarkable feature of the *Azolla*-*Anabaena azollae* symbiosis is the very high frequency of