The utilisation of *Azolla filiculoides* Lam. as a biofertiliser under dryland conditions

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But other seed fell on good ground and yielded a crop that sprang up, increased and produced: some thirtyfold, some sixty and some a hundred

Mark 4: 8

TO MY MUM, MRS. JOYCE KIGULI

and

TO MY UNCLE AND GUARDIAN

THE LATE JAMES NSABIRWA MUKASA

1947-1997

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DEFINITION OF ABBREVIATIONS

CLA	Cumulative Leaf areas
GLA	Green Leaf areas
LA	Leaf Area
LAR	Leaf Area Ratio
RGR	Relative Growth Rate
SLA	Specific Leaf Area
S:R	Shoot to Root ratios
SD20	Sand fertilised with 20% dried *Azolla*
SD50	Sand fertilised with 50% dried *Azolla*
SD80	Sand fertilised with 80% dried *Azolla*
SF20	Sand fertilised with 20% fresh *Azolla*
SF50	Sand fertilised with 50% fresh *Azolla*
SF80	Sand fertilised with 80% fresh *Azolla*
SH20	Sand fertilised with 20% heated *Azolla*
SH50	Sand fertilised with 50% heated *Azolla*
SH80	Sand fertilised with 80% heated *Azolla*
SC	Sand control
S20	Sand fertilised with 20% dried *Azolla*
S80	Sand fertilised with 80% dried *Azolla*
SNPK	Sand fertilised with NPK fertiliser
TC	Topsoil control
T20	Topsoil fertilised with 20% dried *Azolla*
T80	Topsoil fertilised with 80% dried *Azolla*
TNPK	Topsoil fertilised with NPK fertiliser
SE	Standard error

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ABSTRACT

The response of wheat to soil fertilised with varying quantities of the water fern *Azolla filiculoides* was investigated. Experiments were conducted to differentiate between the effects of increased soil mineral status and water status. In the preliminary investigation, experiments were carried out in the greenhouse using potted wheat grown in sand with varying proportions of *A. filiculoides* that had been subjected to various pre-treatments. The pre-treatments were fresh, dry and heated *A. filiculoides* applied at 20%, 50% and 80% volume per 3000 ml. There were significant differences in the measured growth parameters between the plants grown in the various treatments. In addition, the grain yield of wheat plants varied with the different treatments. Results of the preliminary study showed that the addition of heated and dried *A. filiculoides* resulted in significantly better growth than the addition of fresh *A. filiculoides* in sand. For fresh biomass, grain weights, Leaf area ratio (LAR) and relative growth rate (RGR), the performance of dried *A. filiculoides* was as good as that of the heated *A. filiculoides*. Productivity of wheat in the heated treatments increased significantly with increasing proportion of *A. filiculoides* added to sand, while in dry treatments there were no significant increases in productivity in the preliminary study. This supported the hypothesis that *A. filiculoides*, a notorious water weed can be put to agricultural use under dryland conditions in poor nutrient soils. Further investigations using dried *A. filiculoides* in sand and topsoil showed that the use of the same amounts of the dried fern made no significant short term impact on topsoil grown winter wheat but significantly improved the productivity of wheat in sand. Results showed that the addition of dried 20% *Azolla* to sand improved the soil fertility to levels equalling the quality of the control topsoil, but the addition of 80% *Azolla* to sand led to significantly greater wheat productivity than all other treatments. The addition of dried 20% *Azolla* (8.14×10^3 kg $ha^{-1)}$ in sand produced as much wheat biomass as the addition of the recommended NPK fertiliser (30 kg N ha^{-1}) to sand. A comparison between the topsoil and sand-grown plants showed differences in flowering time but these had no effect on the final grain and above ground biomass.

CHAPTER 1: INTRODUCTION

Today, there is a driving force to increase productivity as well as a desire for sustainability in agriculture (Peoples *et al*., 1995; Wagner, 1997). In order to have sustainable agriculture, a constant supply of nutrients with high crop productivity is needed. For farmers to increase productivity, they must apply methods that replenish soil nutrients removed by crops. This should be on a long term basis so as to sustain farming systems.

Among the factors affecting productivity, nutrient availability is of prime importance because of its effect on key physiological and developmental processes that determine plant growth. Fertilisers are utilised to improve the capacity of the soil to supply the necessary nutrients in an agricultural system and are therefore of great importance. In the less developed countries, many farmers cannot afford inorganic fertilisers. This has led to interest in biofertilisation with emphasis on biological nitrogen fixation (Peoples *et al*., 1995; Wagner, 1997).

For centuries, the red water fern *Azolla filiculoides* Lam. (Azollaceae) has been utilised as a green manure for lowland rice in Vietnam and China (Lumpkin & Plucknett, 1982, Wagner, 1997). Due to a symbiotic relationship with a cyanobacterium, *A. filiculoides* is able to fix atmospheric nitrogen (N) which makes it a potential biological source of N and therefore economically important. Use of this small fern would be appropriate, cheaper, and increase productivity in agriculture. World wide interest in its use gained momentum in the 1970's as a result of the fuel crisis that threatened the production of inorganic N fertiliser. Research carried out on different species and strains of *Azolla* showed increased yields in rice production under flooded conditions (Lumpkin & Plucknett, 1982).

In South Africa, *A. filiculoides* occurs in fresh water bodies from where it can be harvested and utilised by farmers. This provides a basis to investigate possible use of

A. filiculoides under dryland conditions in South Africa.

1.1 Biology of *Azolla filiculoides*

Many biological studies on the identification, agronomic use and control of *A. filiculoides* have been carried out. In this study, the key point is the beneficial effects of *A. filiculoides* as an organic fertiliser. Thus information on where it occurs and how it provides N is necessary so that harvests are made at optimum times for maximum nutrient input into the soil.

A. filiculoides is a small free floating fresh water heterosporous fern. It belongs to the order Salviniales and is divided into two sections. Section Rhizosperma with three floats includes *A. pinnata* R. Br. and *A. nilotica* Decne. ex Mett. while section Euazolla with nine floats includes *A. filiculoides* Lam., *A. mexicana* Presl, *A. caroliniana* Willd. and *A. microphylla* Kaulf. (Lumpkin & Plucknett, 1980; Wagner, 1997).

A. filiculoides has a branched rhizome with alternate leaves and roots that hang into the water. Each leaf consists of two lobes; an aerial dorsal lobe (chlorophyllous) and a partially submerged ventral lobe (colourless and cup shaped for buoyancy (Ashton, 1978).

1.1.1 Symbiosis

It is of interest to note that *A. filiculoides* contains cyanobacteria that make it rival with legumes in N production (Ventura & Watanabe, 1993). A symbiotic relationship exists between an endophytic cyanobacterium (*Anabaena azollae* Strasburger) and *A. filiculoides.* The cyanobacterium lives within the dorsal leaf cavity of the fern (Peters & Mayne, 1974; Ashton & Walmsley, 1976, 1984). The endosymbiont fixes N for both organisms while the fern provides a protected environment and a fixed carbon source that benefits the cyanobacteria (Moore, 1969; Van Hove, 1989; Wagner, 1997).

Anabaena azollae excretes ammonium ions into the leaf cavity. These are assimilated by the fern using glutamine synthetase and converted into amino acids (Ashton, 1982; Kannaiyan, 1993). The *Azolla-Anabaena* relationship was found to fix N better than *Sesbania rostrata* (a legume) and able to release it for rice uptake (Ventura and Watanabe, 1993). This was at a minimum of 30-40 kg N ha^{-1} (Watanabe *et al*., 1991) to 70-110 kg N ha^{-1} (Ventura and Watanabe, 1993). According to Talley and Rains (1980), the highest N yield by the fern is before sporulation and therefore plants should be harvested then for optimum use in agriculture.

1.1.2 Reproduction

In view of the fact that *A. filiculoides* is a weed (Hill, 1999, Henderson, 1999), any use for it has to be balanced against the possibility of further spreading of the weed. It is important to note that *A. filiculoides* reproduces both sexually and asexually and its symbiotic relationship exists throughout all generations (Van Hove, 1989). At the first leaf initial of the ventral lobe, the fern produces two sporocarps. Each megaspore contains a colony of *Anabaena azollae* in the form of akinetes which colonise the sporophyte (Ashton, 1982). Fertilisation occurs within the water and the embryo develops into a sporophyte that floats to the water surface after the growth of the first and second leaf (Ashton 1982). Sporulation is associated with dense mat formation in that spores are only formed when the fronds are multi-layerered (Ashton, 1974; Talley and Rains, 1980; Janes 1998a, b). Asexual reproduction is by fragmentation of the fronds. The mature secondary rhizomes or branches form abscission layers at their bases and these secondary rhizomes break off from the main rhizome. Under suitable field conditions, it can double its mass every 3-5 days (Lumpkin & Plucknett, 1982).

1.1.3 Growth

A. filiculoides is capable of photosynthesising at rates higher than most C4 plants due to a variety of light harvesting pigments in the two symbionts. The *Anabaena azollae*

contains cells known as heterocysts that are specifically responsible for N fixation. It has been found that in non symbiotic species of *Anabaena*, the heterocyst frequency never makes up more than 3 to 5% of the cells and yet in mature *Azolla* leaves, they compose up to more than 30% of the cell numbers of the symbiont (Van Hove, 1989).

Various environmental factors affect the growth of the *Azolla* plant; irradiance, temperature, water depth, pH, nutrient status and heavy metals (Ashton, 1982; Lumpkin & Plucknett, 1980; Janes, 1998b). It grows exponentially at a temperature range of 10°C up to 25°C (Talley & Rains, 1980; Ashton, 1982). The plant may turn from green to red which is thought to be a result of production of anthocyanins due to direct sunlight, low temperatures as shown in *A. filiculoides* (Janes, 1998b) and a lack of nutrients especially P (Lumpkin and Plucknett, 1982, Watanabe & Cholitkul, 1990). Strong sunlight has been shown to impair the light harvesting mechanism in *A. caroliniana* resulting in slow N fixation (Kannaiyan, 1993). All these studies show that, like all other plants, the growth of *Azolla* is reduced under poor environmental conditions which in turn reduces the amount of N available in the fern, making it less efficient as a biofertiliser (Ashton, 1982).

1.2 Distribution and weed status

It is advantageous to have a readily available source of a biofertiliser which saves on costs of labour in planting. *A. filiculoides* is widely distributed as a weed in South Africa especially on still and slow moving fresh water bodies (Ashton & Walmsey, 1984; Hill, 1998, 1999, Henderson, 1999). Originally it was found from southern South America through western North America to Alaska. *Azolla* thrives in tropical, subtropical and warm temperate regions (Wagner, 1997: Lumpkin and Plucknett, 1980). It seems to have been introduced to S. Africa as an ornamental during the nineteenth century (Lumpkin & Plucknett, 1980). In South Africa it was first recorded in the Oorlogspoort River near Colesburg in 1948 and has since been recorded at 64 localities by the National Botanical Institute (Hill, 1998).

In South Africa, *A. filiculoides*, produces dense mats (5-30 cm thick) which result in reduction of drinking water quality due to bad odours, coloration and turbidity (Hill, 1999). It increases water borne, water based and water related diseases. It increases evapotranspiration and reduces the water surface area available for recreation and transport. The aquatic biodiversity is also reduced with a specific example being the danger to the Eastern Cape Rocky, *Sandelia bainsii* in the Eastern Cape (Hill, 1998). Beside these factors, it also clogs irrigation pumps, drowns livestock and reduces water flow in irrigation canals (Hill, 1997,1998). Therefore any available means of reducing this environmental hazard is important. A method that is able to reduce the weed as well as utilise it would be ideal. Although *A. filiculoiides* is a weed, the fact that it is a source of N, K, Ca and other nutrients such as Iron and Copper (Jain *et. al*, 1989) makes an investigation of its use for upland (dryland) agriculture worthwhile. It should also be considered as an alternative means of improving soil fertility with long-term effects, in view of the threat of inorganic fertilisers to the environment.

1.3 Controlling *A. filiculoides* infestation

One of the possible means of controlling *Azolla* especially on small water bodies such as dams is mechanical harvesting (Hill, 1998). Not only is this a control mechanism but it also makes the harvested fern available for biofertilisation. Other possible means of control are biological and chemical. Hill (1998) states that mechanical control is ecologically sound whereby infestations of the weed in small accessible areas can be removed with rakes and finely meshed nets. However this facilitates reinfestation under ideal conditions and is labour intensive (Hill, 1999).

Biological control is a sustainable and ecologically benign method. To ensure effective biocontrol, a host-specific organism is needed. In late 1995, the weevil *Stenopelmus rufinasus* was imported from Florida into South Africa and tested for host specificity by the Weeds Division of Plant Protection Research Institute, Pretoria. In its country of

origin, the weevil has not been recorded on any other plant species apart from *A. filiculoides*. Research was carried out on 27 plants in 16 families. Only *A. filiculoides* was found capable of supporting populations of this weevil (Hill 1998). The Directorate of Plant and Quality control of the Department of Agriculture was permitted to release it as a natural enemy for the weed. In December 1997, the weevil was released in South Africa as a biological control for *A. filiculoides* on a dam in the Austin Roberts Bird Sanctuary in Pretoria (Hill 1998). Within two months, the weevils had caused the *Azolla* mat to collapse and after one year there has been no resurgence of the plant even from spores (Hill, 1999). Over the period of January 1998 and March 1999, weevil release at 46 sites all over South Africa, resulted in control of *Azolla* mats at 20 sites and reduction in infestation at five sites (Hill, 1999).

1.4 Why biofertilisation?

1.4.1 Chemical fertilisers

An applicable method of increasing yields is the use of chemical fertilisers which are expensive, disturb the equilibrium of agroecosystems and pollute the environment. This includes pollution of water systems and ground water due to runoff which contains nitrates, acidifies soils and reduces microbial activity (Peoples *et al*., 1995, Wagner, 1997). Most of the chemical nitrogenous fertiliser is produced by industrial N fixation. Each unit of N fertiliser produced requires two units of petroleum (Hamdi, 1982 cited in Wagner, 1997). This is expensive, especially for the farmers in the less developed countries, and petroleum is a non-renewable resource.

1.4.2 Organic fertilisers

In comparison, the application of biofertilisers is inexpensive as it makes use of freely available solar energy, atmospheric N and water. Biofertilisers may be microbial

inoculants or microbially-converted organic materials which are used to supply nutrients to plants (Rengel *et al*., 1999). It is well known that nutrient availability in soil contributes to plant fitness in terms of shoot and root productivity. Concern for the protection of our environment has led to increasing interest in the use of biofertilisers. They are renewable resources, non pollutants, supply other nutrients and improve the general fertility of the soil (Wagner 1997). Biofertilisers promote self sufficiency and reduced costs for resource-poor farmers (Lumpkin & Plucknett, 1982). One of the most limiting elements in agriculture is N and its addition to soil contributes to the dry mass of plants in the form of proteins (Lumpkin & Plucknett, 1982). Most cultivated soils are deficient in N and yet the three most important cereals (wheat, rice and maize) need 20-40 kg soil N ha^{-1} for a period of 3-5 months (Myers, 1988 cited in Peoples *et al*., 1995). Agren (1985), on the assumption that plant growth is determined by the amount of N in the plants, developed a theory on the concept of N productivity in relation to plant nutrition which states that N exerts a strict control over growth and is therefore an essential nutrient.

1.4.3 Mineral nutrients

Apart from N other mineral nutrients are required for plant growth and an increase of these from the deficiency range will increase the growth rate and yield, although the response increases to an optimum after which it reverses and mineral availability interacts with water availability (Marschner, 1995). The number of seeds or fruits and flower initiation can be affected by mineral nutrition which is clearly the case with various micronutrients (Marschner, 1995, Rengel *et al*., 1999). In wheat, copper deficiency affects the reproductive phase as it inhibits anther formation, although it hardly affects the straw (culms and leaves). Boron is essential for pollen tube formation and low supply inhibits flowering and seed production. A limited supply of mineral nutrients such as N, K, P and Mg that are available for re-translocation from source to sink may affect grain yield rather than limited carbohydrate source. Concentrations of Zn and Fe in cereal grain increase with increase of fertiliser (Marschner, 1995). N, P, and K increases shoot and root development and uptake of

other nutrients. *Azolla* may supply micronutrients such as Zn to plants if used as a slow release organic fertiliser (Rengel *et al*., 1999). Short term experiments conducted in India, in the wet season, when inorganic fertiliser and green manure *Azolla* were combined, showed that the rice yield and agronomic efficiency was greater than for inorganic fertiliser alone (Mubarik, 1999).

1.5 Utilisation of *Azolla* as a nitrogen source

Azolla been utilised as a green manure in rice paddies in China and Vietnam for over 2000 years. It is reported to have been first domesticated and used by a peasant woman (Balteng) in Lan Van village in Vietnam (Wagner, 1997, Lumpkin & Plucknett, 1980). During the mid 1960s, the Vietnamese government renewed efforts to extend the area under *Azolla* cultivation. In China it was reported that *Azolla* as a green manure decreased specific gravity and increased porosity and organic matter in lowland soils. *Azolla* is applicable as a biofertiliser because of its endogenous supply of fixed N (Kannaiyan, 1993). The *Azolla-Anabaena* symbiosis has a high productivity and fixes N at high rates (Wagner 1997) which makes it applicable as a biofertiliser in agriculture to supply a natural source of crucial N. The symbiont is able to release nutrients into the soil availing them for plant uptake (Watanabe & Liu, 1992). As fresh material, it has mainly been applied to lowland rice and it has been found to improve soil structure, decompose rapidly, accumulate K in low K environments and grow rapidly (Ashton 1982; Van Hove, 1989). *A. filiculoides* when decaying releases nutrients better as fresh matter than desiccated matter in water (Marwaha *et al*., 1992, Lumpkin & Plucknett, 1982). The decomposition rate depends on the C/N ratio, temperature and soil properties (Marwaha *et al*., 1992; Lumpkin & Plucknett, 1982). The *Azolla* biomass is readily decomposed in flooded rice conditions. It has a favourable C:N ratio of 8-17. The addition of organic matter to the soil narrows the C/N ratio (Kannaiyan, 1993; Ram *et al*., 1994). Ram *et al*. (1994) showed that addition of *Azolla* decreased pH, increased physical soil properties such us aggregation of soil particles, soil structure and permeability leading to better water holding capacity and

less evapotranspiration in sandy loam rice soils. When compared to inorganic fertilisers, the green manure had positive residual effects (Ventura & Watanabe, 1993). The application of *Azolla* increases the N, P and K content of the soil (Lumpkin & Plucknett, 1982, Singh & Singh, 1990; Kannaiyan, 1993).

In Niger the addition of fresh *A. pinnata* to soil increased the tiller, panicle and grain yield of rice (Kondo *et al*., 1989). In a 2 year experiment, carried out in India, *Azolla* (40 kg N ha^{-1}) mixed with *Sesbania* (40 kg N ha^{-1}) was applied to a low land rice-wheat cropping with increase in yields equivalent to the application of 80 kg urea N ha^{-1} (Mahapatra & Sharma, 1989).

Lumpkin and Plucknett (1982) state that the introduction of *A. filiculoides* to China greatly improved rice production with the addition of 30-40 N ha^{-1}. This would be ideal for situations where low cost is required, such as saving on the use of commercial fertilisers. It is important to note that the use of biological N fixation will be affected by environmental factors and the effective use of available N by the crops.
Robinson *et al*. (1991) found that the nutrient uptake by a crop depends on the extent to which the supply of ions from the soil can match the demand for nutrients created by growth. The gradual release of nutrients by decomposition of organic fertilisers is suitable for crop growing and does not have the leaching problems associated with inorganic fertilisers used on sandy low organic matter soils (Becker *et al*., 1995). Lumpkin and Plucknet (1982) state that the addition of *Azolla* benefits poor soils more than good soils. Talley and Rains (1980) state that dried *Azolla* increased yields to the same degree as application of ammonium sulphate at the rates of 40 and 80 kg N ha^{-1}. In a 3 year study, Kolhe and Mittra (1990) showed that *Azolla* can be utilised as a substitute to inorganic N with a residual effect of 63% on wheat over the control in a rice-wheat cropping. Ram and Prasad (1982) found that application and incorporation of *Azolla* (60, 80, 100 tons ha^{-1}) into soil 15 days before sowing had a superior effect on wheat than the application of NPK (40, 40, 10ha^{-1}) which indicated that the *Azolla* must have contributed to soil nutrient status. These studies all confirm that the addition of *A. filiculoides* improves soil fertility and leads to better yields showing that

the nutrients released by the fern are availed to the plants for uptake and growth.

Previous studies show that *Azolla*, especially under lowland flooded conditions, has a positive effect on crop yields since it provides one of the most essential nutrients (N) needed in agriculture and enables uptake of other nutrients. In some cases the residual effect of the *Azolla* on rice has been utilised to increase yields in wheat. While a few studies have been done on the use of fresh *Azolla pinnata* as a biofertiliser of wheat, no comparison has been made on the short term and direct use of heated, fresh and dry *Azolla filiculoides* in dryland agriculture. It was hypothesised that *A. filiculoides*, a notorious water weed in South Africa, can be put to agricultural use under dryland conditions. Therefore, in a preliminary study, the response of wheat to the application of the different treatments of *A. filiculoides* to sand was investigated under greenhouse conditions. Findings of the preliminary experiment allowed for the investigations to be carried out using *Azolla*, sand and topsoil in comparison to the performance of wheat fertilised with inorganic fertilisers.

1.6 Aim of study

To investigate the effect of different treatments of *A. filiculoides* on wheat productivity when used as a biofertiliser under controlled conditions. The *Azolla* biomass was used as fresh, heated, sun and air dried material and observations made on the response of the wheat plants to the fresh and pre-treated *Azolla* under dryland conditions.

1.6.1 Objectives

1. To compare the effect of various treatments of *A. filiculoides* on wheat yield when applied in certain quantities as per volume of sand or topsoil.
2. To determine the amount of *A. filiculoides* needed to improve soil fertility per unit

volume of soil.

3. To determine whether productivity of the plants is improved by the addition of nutrients or improved soil water status.

4. To ascertain whether the use of *Azolla* as a biofertiliser can result in the spread of this water weed.

CHAPTER 2: PRELIMINARY EXPERIMENT

In measuring the bioproductivity of an agricultural crop, biological yield (above ground dry biomass) is of major concern (Beadle, 1993) with specific interest in the economic yield (grain). McDonald (1992) states that early in the season, water availability is not a major constraint and in the absence of weeds and disease, dry matter responses will largely reflect the availability of soil N during this time. The presence of large amounts of mineral N in the soil at the start of the season promote early growth and water use but can also result in depletion of soil moisture (McNeal *et al*., 1971, MacDonald, 1992). In winter wheat, Austin *et al*., (1977) found a strong correlation between dry matter accumulation and N which are affected by photosynthetic carbon fixation. This suggests that measurements of growth parameters such as above ground matter with emphasis on leaves are essential. These are discussed in the following study in relation to *Azolla* applied to pot grown spring wheat.

This preliminary study was undertaken to enable the designing of relevant experiments to evaluate the short term effects of *A. filiculoides* on spring wheat (*Triticum aestivum*, cv Adam Tas) productivity under controlled conditions. The following specific aspects were investigated.

i) The growth response of wheat grown in a variety of *A. filiculoides* fertilised sand as measured by a range of parameters.

ii) The effects of different pre-treatments of *A. filiculoides* on wheat biomass and other growth parameters.

iii) To determine if increasing levels of *A. filiculoides* applied per volume of sand resulted in similar effects on wheat productivity within each treatment.

iv) To determine if the *A. filiculoides* increased the soil water status of the sand.

v) After the harvesting of wheat plants, all treatments were investigated for sporulation to determine if the use of the fern would lead to the spread of *A. filiculoides* as a weed.

2.1 Materials and Methods

2.1.1 Preparation of growth medium

A. filiculoides was harvested using a wire mesh net from a dam on Strowan farm in Grahamstown. River sand was collected from a nearby river bank and acid washed using 10% hydrochloric acid followed by washing with water until the pH was neutral. The pH was tested using a pH meter (cyberscan 1000).

Azolla was subjected to various treatments before being added to the sand. Some of the fern was used as fresh material (F) while the rest was either oven dried (D) at 60°C or sealed in black plastic bin bags and heated (H) in the sun for a week. 1000 cm^3 of fresh *Azolla* weighs 688 g which is reduced to 42.6 g when dried. After heating, the 688 g of fresh *Azolla* weighed 561.3 g indicating fermentation loss during the heating process. Using these figures, the relevant quantities of *Azolla* required for each treatment were calculated as shown in Table 1.

The different treatments (fresh (F); dry (D); heated (H)) of the fern were applied to acid washed sand (S) in varying percentages of 20% (SF20, SD20, SH20), 50% (SF50, SD50, SH50) and 80% (SF80, SD80, SH80) relative to the sand in each pot. Percentages were calculated on a volume per volume basis as shown in Table 1. The control (SC) consisted of pure acid washed sand. Each treatment had 6 replicate pots.

Table 1. Amounts of *Azolla* used to improve the fertility of the sand. Pot dimension is as follows: volume = 3000 cm^3, depth = 15c m, area = 314.16 cm^2. Data are means of three replicates.

Treatment	Mass of fresh *Azolla* added (g pot^{-1})	Density of fresh *Azolla* added per pot g cm^{-3}	Mass of dry *Azolla* added (g pot^{-1})	Density of dry *Azolla* added per pot g cm^{-3}	Mass of heated *Azolla* added (g pot^{-1})	Density of heated *Azolla* added per pot g cm^{-3}
20% (600 cm^{-3})	412.8	0.1376	25.56	0.0085	336.78	0.1123
50% (1500 cm^{-3})	1032	0.3440	63.9	0.0213	841.95	0.2807
80% (2400 cm^{-3})	1651.2	0.5504	102.24	0.0341	1347.12	0.4490

Assuming that fresh *Azolla* contains 0.2% N (Watanabe, 1987), the application of N within the fresh treatments would be as follows; SF20 (0.8256 g N), SF50 (2.064 g N) and SF80 (3.3024 g N) per pot.

A split pot experiment was designed to compare the root biomass partitioning between the control and treated *Azolla* and sand growth medium. The pots had a diameter of 20 cm. Each of the pots was partitioned into two with a plastic plane, 10 cm long (Diagram 1). The control had pure acid washed sand on both sides of the partition. The two treatments had either 80% *Azolla* to sand placed into one side and pure acid washed sand placed in the other side, or had 80% *Azolla* on both sides of the pot. Each pot was evenly topped with pure acid washed sand to a height of 0.5 cm above

the partition. Each treatment was replicated three times.

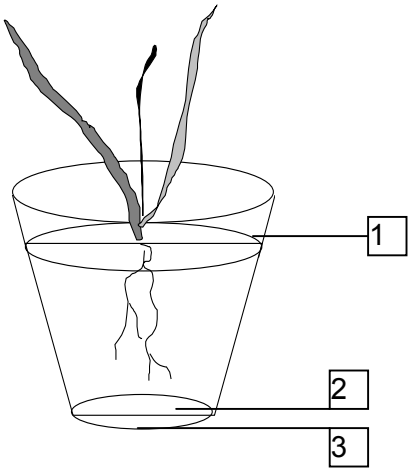

Diagram 1. Split pot design. Compartments; 1 was filled with pure sand, 2 and 3 are the partitions in the pot.

The growth media mixtures in the pots were left for one week in the greenhouse before planting with wheat.

2.1.2 Preparation of wheat seeds

Spring wheat crop seeds were obtained from the Humansdorp Co-op. Wheat seeds (*Triticum aestivum* L. cv Adam Tas) were pre-germinated prior to planting. This was done by placing seeds overnight on moistened filter paper in a Petri dish in the dark. Six seeds were then sown to a depth of 3-4 cm per pot containing the various mixtures of *Azolla* and acid washed sand. A week after germination, plants were culled to five plants per pot.

In the split pot experiment, the seeds were planted directly above the plastic partition and in line with it. After one week plants were culled down to three per pot.

2.1.3 Growth conditions

Potted wheat was grown during the 1998 winter. Treatments were randomly placed in greenhouse and replicates moved around constantly to ensure an even distribution of resources such as light. The lowest temperatures recorded were 10°C and the highest were 25°C for an 12 hour day length and 12 hour nights. Plants were watered daily during the first week and watered after every four days until harvesting at 23, 52 and 78 days.

2.1.4 Plant harvesting and measuring

The effects of biofertilisation with *Azolla* on wheat were recorded as various growth parameters. Two pots of each treatment were harvested at day 23, 52 and 78 and the following growth parameters measured.

Above ground biomass

Plants were cut at the soil surface and their individual fresh weights measured in grams to give the total fresh above ground biomass.

Leaf blade area:

The width and length of each leaf was measured. During the first harvest, these same leaves were photocopied and the photocopies weighed. The surface area of 1g of paper was calculated. This was used to calculate the area of the photocopied leaf as shown in the following equation

$$\text{i) } LA = (PSA \times PLW)$$

where LA is the surface leaf area of each paper leaf which is assumed to be the same as the actual leaf

PSA is surface area of 1g of paper

PLW is the weight of each photocopied paper leaf

The surface area obtained using the length and width was related to that obtained from the photocopied leaves using a linear regression (data was means of 10 plants). The regression equation below was used in the measurement of leaf areas (y) during the rest of the experiment.

ii) [$y= (0.9563x) + 4.9194, R^2 =0.97$]

Leaf area ratio:

For each harvest, leaf blade area (LA) measured was used to calculate leaf area ratio (LAR, Beadle, 1995, Wilhelm, 1998) which is the ratio of the leaf area to the total above ground biomass.

Inflorescence measurements:

At 52 and 78 day harvests, fresh kernel weight, number of spikelets and culm length were also recorded per plant.

Dry weights:

The plants were then oven dried at 70°C for 48 hours and weighed. The dry weights were recorded as total above ground biomass, kernel and grain weights.

Root weights:

For the 78 day harvest, roots were also harvested and used to measure root to shoot dry weight ratios (R:S). For the split pot experiment, the partition was carefully removed and roots from each side separated from the soil. The roots were then oven dried at 70°C and their dry weights recorded.

Relative growth weight:

Relative growth rate (RGR) was calculated as in the following equation (Beadle, 1995).

$$\text{iii) } (\ln W_2 - \ln W_1)/(T_2 - T_1)\ g\ g^{-1}\ day^{-1}$$

W_1 is dry weight at time 1(T_1) and W_2 is dry weight at time 2 (T_2)

2.1.5 Soil water status

The effect of the application of *Azolla* on the soil water status in sand was investigated. After each of the first 3 harvests of the above ground biomass, some of the sand and *Azolla* mixture per pot was placed into smaller pots (12 cm in diameter) for each treatment and watered to full capacity. The pots were then left to drain for 24 hours after which they were measured daily for 25 days. The weight of the soil and water for each day was obtained by subtracting the weight of the pot. The weights were used to calculate percentage soil water content per day as shown in the following equation.

$$\text{iv) } [(W_y - W_x)/ W_y] \times 100.$$

W_x is the final weight of the soil at day 25

W_y is the weight of the soil at day n; n = 1 to 25 days

2.1.6 Viability of spores

After the 23rd day harvest, a small portion of each sand/*Azolla* mixture was placed in Petri dishes (7 cm diameter) in the greenhouse for 2-4 months to find out if there are any viable spores after harvesting of the wheat plants. The growth medium was kept wet with rain water throughout this experiment to ensure a proper environment for the germination of the *Azolla.*

2.1.7 Data analysis

All data was statistically analysed using the General linear model ANOVA at a critical p-level of 0.05 with repeated measures in Statistica, version 1999 (StaSoft. Inc). The treatments and various percentages of the Sand/*Azolla* mixtures were fixed effects with the growth parameters as variables. The Tukey HSD test was used to test for specific effect within the treatments and amounts of *Azolla* added as well as for any interaction effects between the treatments and the increasing proportion of *Azolla* to sand.

2.2 Results

2.2.1 Productivity

To show the effect of biofertilisation with *A. filiculoides* on wheat productivity, each growth parameter mentioned in section 2.1.4 will be discussed in the following section. Observations showed that addition of the different *Azolla* treatments to the sand had improved the growth of the wheat plants. Most trends showed that an increase in proportions of the biofertiliser relative to sand, within a particular treatment, had no significant effect on growth parameters. All growth parameters at the day 23 harvest showed no significant differences between the treatments regardless of the amount of *Azolla* present in the sand. Growth paramaters measured were LA, LAR, fresh and dry biomass. Therefore the data at the 23 day harvest is not shown.

2.2.1.1 Fresh biomass

The three treatments, along with the varying percentages of *Azolla* had an effect over time on the fresh biomass (Figs. 1A &1B, Table 2) of the plants. At day 52, the fresh biomass of the plants responded differently to the increasing volumes of the *Azolla* in

sand.

Effect of the heated *Azolla* treatment on fresh biomass of spring wheat

At day 52, the SC plants had a significantly lower value of fresh biomass than the SH20, SH50 and SH80 plants (Fig. 1A, Table 2). At day 78 (Fig. 1B, Table 2) the fresh biomass of the SC plants was still significantly lower than that of plants harvested from SH20, SH50 and SH80 which showed no significant differences from one another.

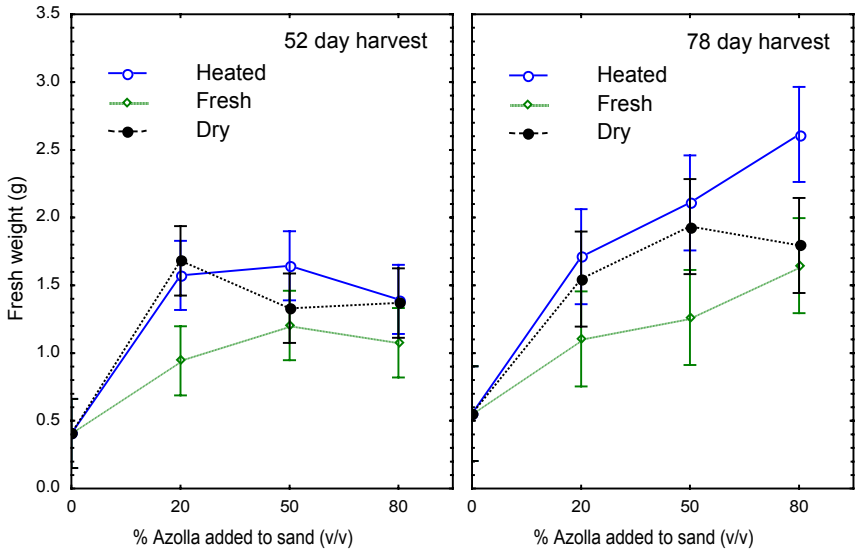

Fig. 1. Fresh biomass harvested from the various treatments at the 52 (A) and 78 (B) day harvests. Data are means (n = 10) with SE.

Table 2. ANOVA results showing the effect of the three treatments of sand and *Azolla* on fresh biomass of wheat at the 52 and 78 day harvests. Values are means of 10 plants followed by letters (a & b) to denote homogeneous groups at a critical p-level of 0.05.

Treatment	% *Azolla* (v/v)	Fresh biomass (g plant^{-1}) (52 days)	Fresh biomass (g plant^{-1}) (78 days)
Heated	0	0.41 a	0.55 a
	20	1.57 b	1.71 b
	50	1.64 b	2.11 b
	80	1.40 b	2.61 b
Fresh	0	0.41 a	0.55 a
	20	0.69 ab	1.10 ab
	50	1.08 b	1.26 b
	80	1.06 b	1.65 b
Dry	0	0.41 a	0.55 a
	20	1.68 b	1.55 b
	50	1.33 b	1.93 b
	80	1.37 b	1.80 b

Effect of fresh *Azolla* treatment on fresh biomass of spring wheat

The general trend was that the fresh *Azolla* positively affected the fresh biomass of the wheat plants. At 52 days (Fig. 1A, Table 2), the fresh biomass of SF50 and SF80 plants was significantly higher than that of SC plants. However, plants harvested from SF20 had greater fresh biomass than SC grown plants but less than SF50 and SF80, although not significantly different from either. At the 78 day harvest (Fig. 1B, Table 2), fresh biomass of SF50 and SF80 plants were significantly higher than those of SC. There was no significant difference on fresh biomass by increasing the proportion of *Azolla* in sand.

Effect of dry *Azolla* treatment on fresh biomass of spring wheat

At the 52 day harvest (Fig. 1A, Table 2), the fresh biomass of all the plants harvested from each of the dry *Azolla* treatments (SD20, SD50 & SD80) was significantly higher than that of SC plants. Plants in all the dry *Azolla* treatments showed no significant difference in fresh biomass between them. At 78 days (Fig. 1B, Table 2), the SD20, SD50 and SD80 plant fresh biomass were significantly higher than the SC plant fresh

biomass. At day 78, there was no significant difference in the response of fresh biomass of plants harvested from SD20, SD50 and SD80.

Comparison between the heated, fresh, dry *Azolla* treatments

Table 3. ANOVA results showing the effect of the three treatments of sand and *Azolla* at three levels of biofertilisation on fresh biomass of wheat at the 52 and 78 day harvests. Values are means of 10 plants followed by letters (a & b) to denote homogeneous groups at a critical p-level of 0.05.

% *Azolla* (v/v)	*Azolla* Treatment	Fresh biomass (g plant^{-1}) (52 days)	Fresh biomass (g plant^{-1}) (78 days)
20	Heated	1.58 a	1.71 a
	Fresh	0.69 b	1.10 a
	Dry	1.08 a	1.55 a
50	Heated	1.64 a	2.11 a
	Fresh	1.08 a	1.26 b
	Dry	1.33 a	1.93 ab
80	Heated	1.40 a	2.61 a
	Fresh	1.08 a	1.65 b
	Dry	1.40 a	1.80 b

At each fertiliser rate, the heated and dry treatments had the best effect on fresh biomass followed by the fresh treatment and the control had the poorest effect.
At the 52 day harvest, fresh biomass of SH 20 and SD20 plants was significantly higher than that of the SF20 plants (Fig. 1A, Table 3). For 50% *Azolla* in sand, there was no significant difference in the fresh biomass between the treatments. At day 52, the fresh biomass of plants in all 80% *Azolla* treatments showed no significant differences between them.

Figure 1B shows the differences in wheat fresh biomass response between the different *Azolla* treatments for each of the increasing *Azolla* proportions per volume of sand at the 78 day harvest. The SH20, SF20 and SD20 plants showed no significant

difference (Table 3) in fresh biomass. The SH50 plant fresh biomass was significantly higher than the SF50 plant fresh biomass. Plants grown in SD50 had a higher fresh biomass than the SF50. At the 78 day harvest, the fresh biomass of SH80 grown plants was significantly higher than that of plants harvested from SF80 and SD80.

2.2.1.2 Dry biomass

Effect of the heated *Azolla* treatment on dry biomass of spring wheat

The use of sand with heated *Azolla* as a growth medium resulted in improved dry biomass of plants. At day 52, dry biomass (Fig. 2, Table 4) of the plants responded differently to the increasing volumes of the fern in sand. The SC plants had a significantly lower value of dry biomass than the SH20, SH50 and SH80 plants. At day 78 (Fig. 2, Table 4), the dry biomass of the SH80 plants was significantly higher than that of plants harvested from SH20, SH50 and SC treatments. All plants harvested from the heated treatments had significantly higher biomass than the SC plants.

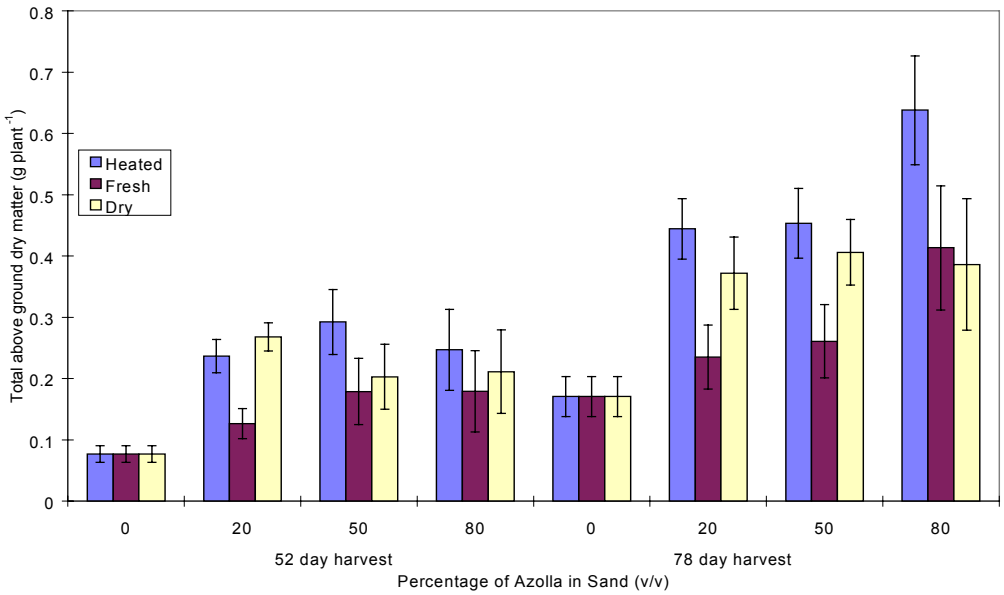

Fig. 2. The dry biomass of plants harvested at 52 and 78 days from the control and

the different sand with *Azolla* treatments. Data are means of 10 plants with SE.

Effect of fresh *Azolla* treatment on dry biomass of spring wheat

Although the effect of the sand with fresh *Azolla* growth medium was not as high as that of the sand with heated *Azolla*, it was still notable in comparison to the control. At 52 days (Fig. 2, Table 4), the dry biomass of SF80 and SF50 was significantly higher than that of SC plants but not significantly different from the dry biomass of SF20 grown plants. At the 78 day harvest (Fig. 2, Table 4), dry biomass of the SF80 plants was significantly higher than SF20 and SC plants but not significantly different from SF50 grown plants. At the 78 day harvest, a comparison of the fresh treatments showed the dry biomass response to be different from the fresh biomass response. The dry biomass of SF80 grown plants is significantly higher than that of the SF20 grown plants unlike the fresh biomass response which is the same for both treatments. This suggests that SF20 treatment contributed to more plant water content than SF50 and SF80 treatments.

Effect of dry *Azolla* treatment on dry biomass of spring wheat

In terms of total above ground dry biomass, the sand with dry *Azolla* growth medium resulted in a better response than the control. At the 52 day harvest (Fig. 2, Table 4), the dry biomass of all the plants harvested from each of the other dry *Azolla* treatments (SD20, SD50 & SD80) were significantly higher than SC plants. At 78 days (Fig. 2, Table 4), the dry biomass of plants harvested from the SD20, SD50 and SD80 treatments were significantly higher than the SC plant dry biomass.

Table 4. ANOVA results showing the effect of the three treatments of sand and *Azolla* on dry biomass of wheat at the 52 and 78 day harvests. Values are means of 10 plants followed by letters (a, b & c) to denote homogeneous groups at a critical p-level of 0.05.

Treatment	% *Azolla* (v/v)	Dry biomass (g plant^{-1}) (52 days)	Dry biomass (g plant^{-1}) (78 days)
Heated	0	0.08 a	0.17 a
	20	0.24 b	0.44 b
	50	0.29 b	0.45 b
	80	0.25 b	0.64 c
Fresh	0	0.08 a	0.17 a
	20	0.13 b	0.24 ab
	50	0.18 b	0.26 bc
	80	0.18 b	0.41 c
Dry	0	0.08 a	0.17 a
	20	0.27 b	0.37 b
	50	0.20 b	0.41 b
	80	0.21 b	0.39 b

Comparison between the heated, fresh and dry *Azolla* treatments

Pre-treatment of *Azolla* had significant effects on the response of plant dry biomass over time. At the 52 day harvest, dry biomass of SH20 and SD20 plants was significantly higher than that of the SF20 plants (Fig. 2, Table 5). SH50 plant dry biomass was significantly higher than SF50 plant dry biomass. However, SD50 plant dry biomass was not significantly different from either (Fig. 2, Table 5). At day 52, the dry biomass of plants in all 80% *Azolla* treatments showed no significant differences between them.

At the 78 day harvest, the plants grown in SH80 had the highest dry biomass which was significant from all other treatments. SD50 and SF80 showed a similar response with dry biomass being significantly higher than that of SC grown plants. The dry biomass trend was different from the fresh biomass trend observed at the 78 day harvest at the 20% fertiliser rate. While there was no difference in fresh biomass, between treatments, there was a difference in dry biomass suggesting a difference in plant water content. SF20 had an increasing effect on the plant water content.

Table 5. ANOVA results showing the effect of the three treatments of sand and *Azolla* at three levels of biofertilisation on dry biomass of wheat at the 52 and 78 day harvest.

Values are means of 10 plants followed by letters (a & b) to denote homogeneous groups at a critical p-level of 0.05.

% *Azolla* (v/v)	*Azolla* Treatment	Dry biomass (g plant^{-1}) (52 days)	Dry biomass (g plant^{-1}) (78 days)
20	Heated	0.24 a	0.44 a
	Fresh	0.13 b	0.24 b
	Dry	0.27 a	0.37 a
50	Heated	0.29 a	0.45 a
	Fresh	0.18 b	0.26 b
	Dry	0.20 ab	0.41 a
80	Heated	0.25 a	0.64 a
	Fresh	0.18 a	0.41 b
	Dry	0.21 a	0.38 b

2.2.1.3 Spikelet, grain numbers and grain weight

Significant differences were observed within the different treatments and increasing percentages of *Azolla* on the spikelet numbers and grain weights (Tables 6 & 7). Spikelet numbers are important since they contribute to the amount of grain per plant. A representative, randomly chosen sample of spikes was dissected and there were eight florets observed per spikelet. On each spikelet, every fourth floret was not fertilised. The grain obtained was only a result of fertilisation of the 2-3 outermost florets per spikelet. However some of the florets aborted resulting in production of only one grain per spikelet and therefore less grain per plant.

Table 6. ANOVA results showing the effect of the three treatments of sand and *Azolla* on spikelet, grain numbers and grain dry weight of wheat at the 52 and 78 day harvests. Values are means of 10 plants with letters (a, b & c) to denote homogeneous groups at a critical p-level 0.05.

Treatment	% *Azolla* (v/v)	Spikelets No. spike^{-1} (52 days)	Caryopses No. spike^{-1} (78 days)	Grain weight g plant^{-1} (78 days)
Heated	0	3.1 a	3.7 a	0.06 a
	20	8.7 b	10.3 b	0.16 b
	50	8.7 b	10.9 b	0.17 b
	80	6.4 b	16.6 c	0.23 b
Fresh	0	3.1 a	3.7 a	0.06 a
	20	2.5 a	7.2 b	0.07 a
	50	7.7 b	7.5 b	0.08 ab
	80	5.6 ab	11.6 b	0.16 b
Dry	0	3.1 a	3.7 a	0.06 a
	20	6.8 b	9.1 b	0.17 b
	50	3.7 a	9.5 b	0.14 b
	80	6.5 b	8.1 b	0.15 b

Effect of heated *Azolla* treatment

By the time of the second harvest, most of the plants had flowered. At the 52 day harvest (Table 6), the number of spikelets was significantly higher on the SH20, SH50 and SH80 plants than the SC plants. At the 78 day harvest (Table 6), the trend shown by grain numbers reflected that of spikelet numbers at day 52. The grain numbers of SH20, SH50 and SH80 plants were significantly higher than those of the SC plants. Within the fertiliser treatments, the grain numbers of SH80 grown plants were significantly greater than those of SH20 and SH50 plants. Therefore, the number of florets that were initiated was much higher on the SH80 plants than all the other plants.

The grain weights (Table 6) reflected the same trend as the grain numbers. The three fertiliser treatments (SH20, SH50 & SH80) resulted in significantly higher grain weight than the SC treatment.

Effect of fresh *Azolla* treatment

At the 52 day harvest (Table 6), the SF50 plants had significantly higher number of spikelets than the SF20 and SC plants. The number of spikelets per plant in SF20 plants was very low because fewer plants had spikes at this stage. At the 78 day harvest (Table 6), when all grain had fully formed, the grain numbers of plants grown in all fresh *Azolla* treatments were significantly higher than those of plants grown in SC treatment. However the grain weights of plants grown in SF80 were significantly greater than those of SC and SF20 grown plants and the same as those of plants harvested from the SH50 treatment.

Within the fresh *Azolla* treatments, the grain weights did not correspond to grain numbers of SF20 and SF50 grown plants (Table 6). The notable difference between SF80 and the other two fresh treatments suggests a slower rate of grain filling in the SF20 and SF50 treatments.

Effect of dry *Azolla* treatment

At the 52 day harvest, the SD20 and SD80 spikelet numbers were significantly higher than those of the SD50 and SC grown plants (Table 6). At the 78 day harvest (Table 6), the grain numbers of plants harvested from the fertilised treatments were significantly higher than those of the SC grown plants.

Within the dry *Azolla* treatments, the dry grain weights of SD20, SD50 and SD80 plants were significantly higher than those of SC plants (Table 6). This is the same the trend as observed in grain numbers.

Comparison between the heated, fresh and dry *Azolla* treatments

Table 7. ANOVA results showing the effect of the three treatments of sand and *Azolla* at three levels of biofertilisation on spikelet, grain numbers and grain dry weights of wheat at the 52 and 78 day harvest. Values are means of 10 plants followed by letters (a & b) to denote homogeneous groups at a critical p-level 0.05.

% *Azolla* (v/v)	*Azolla* Treatment	Spikelets No. spike^{-1} (52 days)	Caryopses No. spike^{-1} (78 days)	Grain weight g plant^{-1} (78 days)
20	Heated	8.7 a	10.3 a	0.16 a
	Fresh	2.5 b	7.2 a	0.07 b
	Dry	6.8 a	9.1 a	0.17 a
50	Heated	8.7 a	10.9 a	0.17 a
	Fresh	7.7 a	7.5 a	0.08 b
	Dry	3.7 b	9.5 a	0.14 ab
80	Heated	6.4 a	16.6 a	0.23 a
	Fresh	5.6 a	11.6 b	0.16 b
	Dry	6.5 a	8.1 b	0.15 b

The general trend shown between treatments is that the plants grown in the sand with heated and dry *Azolla* treatments had higher spikelet numbers and grain weights (Table 7). The spikelet numbers of plants grown in SD20 and SD80 were the same as those of plants grown in the SF50 and SF80 treatments. Spikelet numbers of SD50 and SF20grown plants were the lowest (Table 6 & 7). The grain weights of SH20 and SD20 plants were significantly higher than those of SF20 plants. At proportions of 50% *Azolla* in sand, the plants grown in SH50 had significantly higher grain weights than those from SF50 pots. Grain weights of SH50 grown plants were greater but not significantly different from those of plants harvested from SD50 pots. SH80 plants had a significantly higher grain weight than plants grown in SF80 and SD80 treatments. Plants grown in SH50, SH80 and SD20 treatments produced the best grain weights per plant.

2.2.1.4 Culm length

Table 8. ANOVA results showing the effect of the three treatments of sand and *Azolla* on the culm length of wheat at the 52 and 78 day harvests. Values are means of 10 plants with letters (a & b) to denote homogeneous groups at a critical p-level 0.05.

Treatment	% *Azolla* (v/v)	Culm length (cm) (52 days)	Culm length (cm) (78 days)
Heated	0	13.93 a	11.96 a
	20	16.39 a	18.98 b
	50	17.81 a	21.41 b
	80	10.47 a	20.7 b
Fresh	0	13.93 a	11.96 a
	20	2.88 b	9.01 a
	50	9.95 a	10.79 a
	80	8.8 a	14.32 a
Dry	0	13.93 a	11.96 a
	20	20.05 b	17.79 b
	50	11.29 a	15.35 ab
	80	15.44 b	14.97 ab

Both treatment (Table 8) and quantity of *Azolla* added (Tables 9) affected culm length which thus had an effect on plant height. Culm length alone was not a very sensitive growth parameter.

Effect of heated *Azolla* treatment

At the 52 day harvest, plants harvested from all SH pots did not have a significantly different culm length from that of SC grown plants (Table 8). At the 78 day harvest (Table 8), the SH20, SH50 and SH80 plants' culm length was significantly higher than the culm length of the SC grown plants. This is a similar trend as was mentioned for the grain weights.

Effect of fresh *Azolla* treatment

The trend, at 52 days (Table 8), was different for the fresh treatments of *Azolla*; the SC, SF50 and SF80 plants showed significantly longer culms than the SF20 plants due to the difference in culm growth rate. At the 78 day harvest, within the fresh *Azolla* treatments, there was no significant difference (Table 8) between the culm length of the SC grown plants and that of the SF20, SF50 and SF80 grown plants.

Effect of dry *Azolla* treatment

Within the sand with dry *Azolla* treatments. The harvest at 52 days (Table 8) showed a trend that is different from the other two treatments. The SD20 and SD80 grown plants had a significantly higher culm length than that of the SD50 and SC grown plants. At 78 days, only the SD20 grown plants had significantly higher culm lengths than the SC plants.

Comparison between the heated, fresh and dry *Azolla* treatments

At the final harvest, the SH20 and SD20 grown plants had a significantly higher culm length than the SF20 grown plants (Table 9) which is similar to the grain weights. The SH50 and SD50 grown plants had a significantly lower culm length than the SF50 grown plants. However, plants harvested from the SH80 treatment had a significantly longer culm than the SF80 and SD80 grown plants.

Table 9. ANOVA results showing the effect of the three treatments of sand and *Azolla* on the culm length of wheat at the 78 day harvest between the different treatments at each of the three proportions of *Azolla*. Values are means of 10 plants with letters (a & b) to denote homogeneous groups at a critical p-level of 0.05.

% *Azolla* (v/v)	***Azolla* Treatment**	**Culm length (cm) (52 days)**	**Culm length (cm) (78 days)**
20	Heated	16.39 a	18.98 a
	Fresh	2.88 b	9.01 b
	Dry	20.05 a	17.79 a
50	Heated	17.81 a	21.41 a
	Fresh	9.95 b	10.79 b
	Dry	11.29 ab	15.35 a
80	Heated	10.47 a	20.7 a
	Fresh	8.8 a	14.32 b
	Dry	15.44 a	14.97 b

2.2.1.5 Leaf Area (LA)

The general trend shown by leaf areas (cm^2) in response to the addition of *Azolla* to sand is an increase in leaf surface area (Figs. 3A & 3B, Tables 10 & 11). At day 52 (Fig. 3A, Table 10), all *Azolla* treatments had significantly larger leaf areas per plant than the SC plants. Within the different treatments, increasing the proportions of *Azolla* per volume of sand had no significant effect on the plant leaf areas (Fig. 3A, Table 10) except in the fresh *Azolla* treatments. At the 78 day harvest, the SH20, SH50 and SH80 grown plants had significantly higher leaf areas than SC plants (Fig. 3B, Table 10). However only SF80 grown plants had significantly higher leaf areas than SC plants. The SD20 and SD80 plants had greater leaf areas than SC plants but only SD50 plants had significantly larger leaf areas than the SC plants.

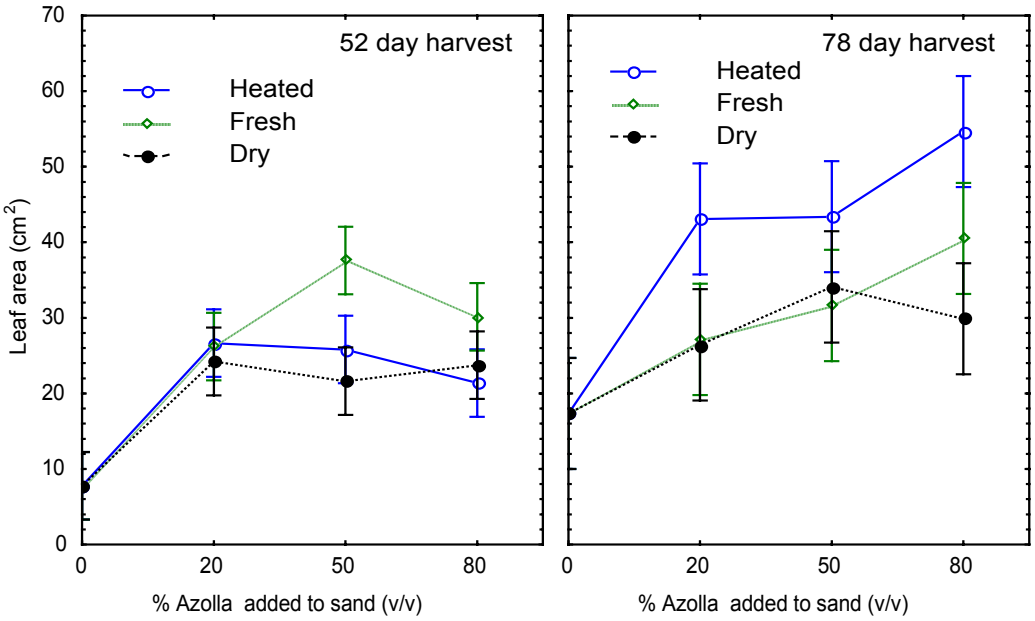

Fig. 3. The leaf surface areas of plants harvested from the various treatments at the 52 (A) and 78 (B) day harvest. Data are means (n = 10) with SE.

Table 10. ANOVA results showing the effect of the three treatments of sand and *Azolla* on leaf area of wheat at the 52 and 78 day harvests. Values are means of 10 plants with letters (a, b & c) to denote homogeneous groups at a critical p-level of 0.05.

Treatment	% *Azolla* (v/v)	Leaf area (cm^2 $plant^{-1}$) (52 days)	Leaf area (cm^2 $plant^{-1}$) (78 days)
Heated	0	7.78 a	17.34 a
	20	26.65 b	43.08 b
	50	25.81 b	43.39 b
	80	21.38 b	54.66 b
Fresh	0	7.78 a	17.34 a
	20	19.87 b	27.16 ab
	50	33.81 c	31.64 ab
	80	30.13 bc	40.51 b
Dry	0	7.78 a	17.34 a
	20	24.623 b	26.43 ab
	50	21.63 b	34.10 b
	80	23.73 b	29.90 ab

Comparison between the heated, fresh and dry *Azolla* treatments

At day 52, a comparison between the different treatments at the 20% and 80% biofertiliser rate showed no significant differences in leaf areas (Fig. 3B, Table 11).

The SF50 grown plants had significantly lower leaf areas than the SH50 and SD50 grown plants. At the 78 day harvest, SH20 grown plants had significantly higher leaf areas than SF20 and SD20 grown plants. There were no significant differences in leaf areas of plants grown at the 50% fertiliser rate. The SD80 grown plants had significantly lower leaf areas than SH80 grown plants.

Table 11. ANOVA results showing the effect of the three treatments of sand and *Azolla* at three levels of biofertilisation on leaf area of wheat at the 52 and 78 day harvest. Values are means of 10 plants followed by letters (a, & b) to denote homogeneous groups at the p-level of 0.05.

% *Azolla* (v/v)	***Azolla* Treatment**	**Leaf area (cm^2 $plant^{-1}$) (52 days)**	**Leaf area (cm^2 $plant^{-1}$) (78 days)**
20	Heated	26.65 a	43.08 a
	Fresh	19.87 a	27.16 b
	Dry	24.63 a	26.43 b
50	Heated	25.81 a	43.39 a
	Fresh	33.81 b	31.64 a
	Dry	21.63 a	34.10 a
80	Heated	21.38 a	54.66 a
	Fresh	30.13 a	40.51 ab
	Dry	23.73 a	29.87 b

2.2.1.6 Leaf Area Ratio (LAR)

The LAR response within each treatment

Figure 4A and Table 12 show that at day 52, there was no significant difference between the LAR of SC grown plants and the LAR of SH20, SH50 and SH80 grown plants. Similarly there was no significant difference between the LAR of SC grown

plants and the LAR of SD20, SD50 and SD80 grown plants. The trend shown by the fresh *Azolla* treatments was different. The LAR of SF20, SF50 and SF80 grown plants was significantly greater than that of SC grown plants.

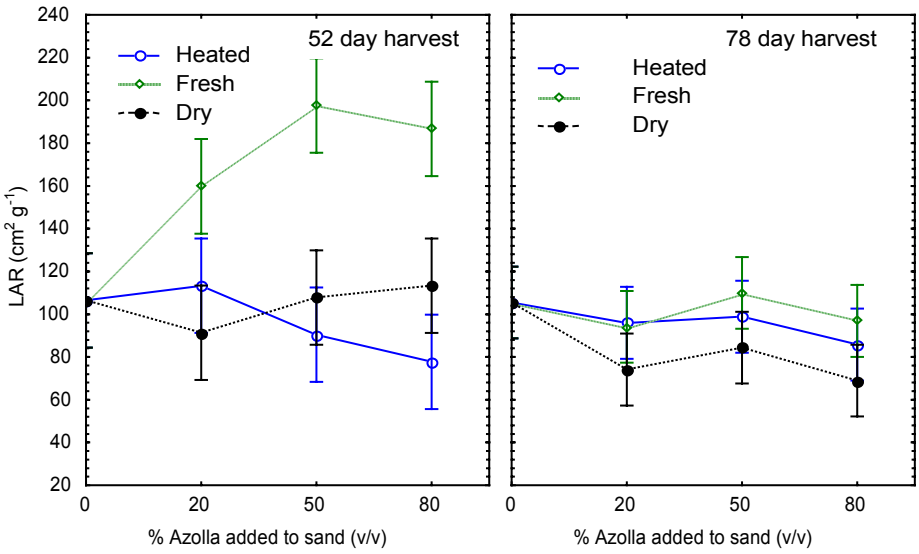

Fig. 4. The Leaf Area Ratio (LAR) of plants harvested from the various treatments at the 52 (A) and 78 (B) day harvest. Data are means (n = 10) with SE.

At day 78 (Fig. 4B, Table 12), the general trend was that there was no significant difference in LAR between all the different fertiliser rates and the control. The LAR of SC grown plants was not significantly different from that of all the plants grown in the fresh *Azolla* treatments (Fig. 4B, Table 12). Within the heated treatments, the increase in fertiliser did not result in a significantly different response in LAR from that calculated for SC grown plants. Both SD20 and SD80 grown plants had a significantly lower value of LAR than SC grown plants (Fig. 4B, Table 12).

Table 12. ANOVA results showing the effect of the three treatments of sand and *Azolla* on leaf area ratio (LAR) of wheat at the 52 and 78 day harvests. Values are means of 10 plants with letters (a & b) to denote homogeneous groups at a p-level of 0.05.

Treatment	% *Azolla* (v/v)	Leaf area ratio (LAR) (52 days)	Leaf area ratio (LAR) (78 days)
Heated	0	106.51 a	105.47 a
	20	114.01 a	94.07 a
	50	90.40 a	98.82 a
	80	77.74 a	85.81 a
Fresh	0	106.51 a	105.47 a
	20	159.80 b	95.97 a
	50	197.65 b	109.96 a
	80	186.63 b	96.89 a
Dry	0	106.51 a	105.47 a
	20	89.64 a	76.60 b
	50	107.84 a	84.39 ab
	80	113.38 a	74.15 b

The LAR response between the different treatments

Treatment hardly affected LAR. At the 52 day harvest, the SF20 grown plants had a higher value of LAR than the SH20 and SD20 grown plants. (Fig. 4A, Table 13). The same trends were observed at the 50% and 80% fertiliser rate between treatments. At day 52, the SH80 treatment had the lowest value on LAR and therefore the best effect. The difference between heated and dry treatments with the increasing percentage of *Azolla* does not seem to vary greatly.

Figure 4B and Table 13 show that at the 78 day harvest, there were no significant differences in LAR between treatments at all the fertiliser rates.

Table 13. ANOVA results showing the effect of the three treatments of sand and *Azolla* at three levels of biofertilisation on leaf area ratio (LAR) of wheat at the 52 and 78 day harvest. Values are means of 10 plants followed by letters (a & b) denote homogeneous groups at a p-level of 0.05.

% *Azolla* (v/v)	*Azolla* Treatment	Leaf area ratio (LAR) (52 days)	Leaf area ratio (LAR) (78 days)
20	Heated	114.01 a	94.07 a
	Fresh	159.80 b	95.97 a
	Dry	89.64 a	76.60 a
50	Heated	90.40 a	98.82 a
	Fresh	197.65 b	109.96 a
	Dry	107.84 a	84.39 a
80	Heated	77.74 a	85.81 a
	Fresh	186.63 b	96.89 a
	Dry	113.38 a	74.15 a

2.2.1.7 Root: shoot dry biomass

The root: shoot dry biomass ratios response within each treatment

The roots were only harvested at 78 days due to difficulties encountered in separating of the roots from the *Azolla* growth media. Both treatment and quantity of *Azolla* added had a significant effect on the root to shoot ratio dry weights (Tables 14 & 15).

Within the heated treatments, SH20 grown plants had significantly higher R:S ratios than the SC, SH50 and SH80 grown plants (Table 14). Within the fresh treatments, there was no significant difference in R:S ratios between SF20, SF80 and SC grown plants. However, R:S ratios of the SF50 plants were significantly lower than those of the SC grown plants. The R:S ratios of plants harvested from SC and dry *Azolla*

treatments showed no significant differences between them.

Table 14. ANOVA results showing the effect of the three treatments of sand and *Azolla* on R:S dry biomass ratios of wheat at the 78 day harvest. Values are means of 10 plants with letters (a & b) to denote homogenous groups to homogeneous groups at a p-level of 0.05.

Treatment	**% *Azolla* (v/v)**	**root: shoot dry weights plant^{-1} (78 days)**
Heated	0	0.15 a
	20	0.22 b
	50	0.11 a
	80	0.08 a
Fresh	0	0.15 a
	20	0.06 ab
	50	0.04 b
	80	0.07 ab
Dry	0	0.15 a
	20	0.09 a
	50	0.13 a
	80	0.09 a

The root to shoot dry biomass ratios response between treatments

Between the treatments, the SH20 had significantly higher R:S ratios than SD20 and SF20 plants (Table 15). The SD50 plants had significantly higher R:S ratios than SF50 plants. The R:S ratios of SH50 were not significantly different from either treatment at the 50% fertiliser rate. However, at the highest biofertiliser rate there was no significant differences between treatments.

Table 15. ANOVA results showing the effect of the three treatments of sand and *Azolla* at three levels of biofertilisation on R:S dry biomass of wheat at the 78 day harvest. Values are means of 10 plants followed by letters (a & b) to denote homogeneous groups at a p-level of 0.05.

% *Azolla* (v/v)	***Azolla* Treatment**	**root:shoot dry weights plant^{-1} (78 days)**
20	Heated	0.21 a
	Fresh	0.06 b
	Dry	0.09 b
50	Heated	0.11 ab
	Fresh	0.04 b
	Dry	0.13 a
80	Heated	0.08 a
	Fresh	0.07 a
	Dry	0.09 a

2.2.1.8 Relative growth rate (RGR)

RGR data is not shown but is discussed briefly. Over the 23-52 days period, there was no significant difference in RGR within each treatment, or between treatments and the control. The same trend was observed over the 52-78 days period.

At each particular fertiliser rate between the treatments, there was no significant difference in RGR. However as expected, there was a decrease in RGR over time in all treatments.

2.2.2 Soil moisture content

Figures 5A to 5C show the percentage water retained within the growth media. The moisture content of the soil decreased over time in the various treatments. Increasing

A. filiculoides per volume of sand from 20% to 50% and to 80% *Azolla* did not have a significant effect on the moisture content in the dry and fresh treatments but from 20% to 80% in the heated treatments showed a significant effect, allowing the soil to retain more moisture.

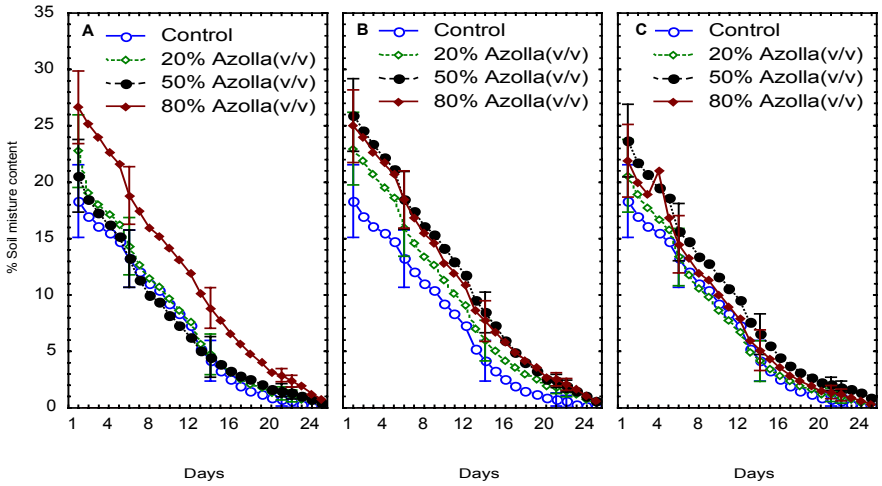

Fig. 5 The percentage water content of sand with heated (A), fresh (B), dry *Azolla* (C) added as biofertiliser in comparison to pure sand growth media. Data are means (n=4) with SE at 1, 6, 14, 21 and 22 days.

Effect of heated *Azolla* treatment

Within the sand with heated *Azolla* treatments, the addition of 80% biofertiliser significantly increased the water retention capacity of the growth medium (Fig. 5A). After day one, the water content in the SH80 pots was significantly greater than the water content retained in the SC and SH50 pots though not different from that in SH20 pots. This was still observable after six days. After 2-3 weeks, the percentage water content retained in the SH80 pots was significantly higher than that in SC, SH20 and SH80 pots.

Effect of fresh *Azolla* treatment

Figure 5B shows the percentage water retained in the fresh fertiliser growth media and the control. The addition of biofertiliser certainly improves the water retention capacity of the sand. After day one, the SF50 and SF80 pots contained significantly more water than the SC pots which did not contain a significantly lower percentage of water than SF20 pots. This same trend is observed after two and three weeks.

Effect of dry *Azolla* treatment

Figure 5C shows the percentage water content in the SC, SD20, SD50 and SD80 pots. The addition of the fertiliser to sand increased the water retention capacity of the sand but this was not significant from the control.

Comparison between the heated, fresh and dry *Azolla* treatments

The percentage water content of SH20, SF20 and SD20 was not significantly different between these treatments (Fig. 5). The general trend showed that SH50 treatment had significantly lower water content than SF50 which was not significantly different from SD50. The SH80 and SF80 treatments had better water retention in comparison to the SD80 treatments without significant differences between them (Fig. 5).

2.2.3 Sporulation

After a period of 4 months, no spore germination was observed in any of the Petri dishes from each of the different treatments.

2.2.4 Split pot experiment

Table 16. Root dry weights collected from each side of the split pots with different treatments. Data are means of 10 with SE.

Treatment	Root weight from fertilised sand (g)	Root weight from control (g)
Control	0.0273 ±0.0063	0.0121±0.0142
Heated *Azolla*	0.0225±0.0053	0.0192±0.0070
Fresh *Azolla*	0.0232±0.0010	0.0010±0.0024
Dry *Azolla*	0.0556±0.0040	0.0103±0.0038

The highest root biomass was observed in the split pot with the dried *Azolla* (Table 16). However, there was difficulty in separating the roots from the *Azolla* which might have affected the heated and fresh *Azolla* grown plants. With all the treatments, less dry root weight was collected from the pure acid washed sand. The control showed similar root weight on each side of the split pot. These observations suggest a higher root growth in the dried *Azolla* treatment.

2.3 Discussion and Summary

In order to facilitate ease of comparison across all growth parameters measured, the best and poorest responses were summarised in Table 17. The heated (SH80) was generally the best and the control (SC) was the poorest as shown in table 17. In most cases, the fresh treatments resulted in a poor performance, after comparison to that of the SC treatment. Although the SH80 had the highest grain weight, the dry *Azolla* treatments had higher average weight per grain (data not shown). Furthermore the plants harvested from the dry treatments produced smaller, heavier leaves and had the best matter accumulated per leaf area. The SH80 had the highest grain numbers and a significantly higher grain weight than the dry treatments.

Table 17. Summary of the best and poorest responses of the growth parameters to various treatments.

Growth parameter	Treatment with best response	Homogenous group members	Treatment with poorest response	Homogenous group members
Fresh biomass	SH80	SH50, SD50	SC	SF20
Dry biomass	SH80	NONE	SC	SF20
Grain numbers	SH80	NONE	SC	NONE
Grain weights	SH80	SH50, SD20	SC	SF20, SF50
Culm length	SH50	SH20, SH80, SD20	SC	SF20, SF50, SF80, SD50, SD80
Leaf area	SH80	SH20, SH50, SF80	SC	SD20, SD80, SF20, SF50
LAR	SD80	SD20, SD50, SH80	SF50	SC, SF20
Root: shoot	SH20	all except SF50	SF50	SF20, SF80, SD20, SD80, SH50, SH80
RGR	SH50	ALL	SD20	ALL

Watanabe and Ito (1985) found that under lowland (flooded) cultivation, fresh *Azolla* material released NH_4-N better than dry material for uptake by rice. The general trends show that the dry and heated treatments resulted in a better effect on most of the growth parameters while the fresh treatment and control are homogenous groups with a poorer response (Table 17). The dry and heated treatments may have had a narrower C/N ratio releasing N more efficiently into the soil than the fresh treatment (Watanabe *et al*., 1991). The difference in nutrient release maybe a result of flooded conditions in comparison to dryland (upland) conditions. Assuming 0.2% N in fresh *Azolla*, (Watanabe, 1987), 0.5504 g cm^{-3} of fresh *Azolla*, containing 3.3024 g N per pot improved yields as much as all the dry treatments, SH20 (0.1123 g cm^{-3}) and SH50 (0.2807 g cm^{-3}) treatments as shown in Figure 2.

The amount of grain produced within the heated and dry treatments was the same irrespective of the amount of fern applied to the sand, but that of SF20 was significantly lower than that of the SF80 treatment (Table 7 & 17). Significant grain increases observed in the dry and heated *Azolla* treatments show the same results.

This was also found in India when *A. pinnata* harvested from natural waters during October to February was used to biofertilise wheat (Prasad & Ram, 1981, Ram and Prasad, 1983). Between the treatments the best grain harvest (Table 17) was obtained from SH80 followed by SD20 and then SF80. These were all significantly better than the grain harvests of SC grown plants (Table 6). Therefore in order to obtain the best above ground matter and grain harvests, the use of SH80 followed by SD20 would be advisable for short term use of *A. filiculodes*. Similar results are shown by the culm length data in that the longest culms were observed in the heated and dry treatments (SH50 and SD20) treatments (Table 17). SH50 grown plants had longer culms than SH80, although not significantly different (Table 17). However, plants with the longest culms do not necessarily produce the best grain and above ground biomass. Therefore culm length should be used along with other growth parameters.

Leaf areas were largest on SH80 grown plants followed by the SF80 and then SD20. An increase in leaf area would lead to an increase in the photosynthetic carbon fixation and thus affect the dry matter accumulation and N present in the plant (Austin *et al*., 1977). To support this, LAR was calculated. Both heated and dry treatments at all levels of *Azolla* application gave lower LAR than the fresh treatments showing that the dry matter accumulation was more efficient in the former two treatments (Fig. 4, Table 12 & 13). Although not significantly better than the heated treatments, the dry *Azolla* treatment still resulted in a more efficient dry matter accumulation per available leaf area than the heated treatment. In this respect, the SD20 treatment performed best since it is showed the least variability. The LAR of plants SD20 grown plants was significantly lower than that of the SC plants.

The lack of difference in RGR despite differences in LAR may have been due to fresh *Azolla* contributing more to vegetative growth while the dry and heated treatments contributed equally to both reproductive and vegetative growth.

The general trend shown by the R:S ratios shows no significant differences between the control and all other treatments which is unexpected since there were observable

differences in shoot biomass. The problem may be attributed to the separation of roots from the *Azolla* mixtures leading to loss of root material. The lowest R:S ratios were observed in SF50 treatments while the highest R:S ratios were found in SH20 grown plants. This suggests that SF50 plants allocated the least biomass to below ground while the SH20 had the best below ground biomass allocation. An increase of the sample size might also give more conclusive results. However, the split pot experiments clearly show a significantly higher root biomass in the dry *Azolla* treatment suggesting a better root development in this treatment.

Moisture holding capacity was significantly improved in SH80, SF50 and SF80 treatments (Fig. 5). Although there was an increase in SD50 moisture content, it was not significant. However if improved yields were entirely the result of improved water status then the fresh treatment would have given yields as good as the dry and heated.

The incorporation of the fern into soil improves physical properties and water holding capacity of the soil (Ram *et al*., 1994) which is reflected by, response of wheat grown in various treatments in comparison to the control. The SF20 treatment seems to have had an effect on the plant water content, although it did not effect improved growth parameters.

Vegetative reproduction by the water fern appears to be impossible when used under dryland conditions and fully incorporated into the soil. The portions of soil placed in water to investigate for viable spores gave negative results suggesting that *A. filiculoides* may only be a threat under flooding conditions whereby the fronds are carried to other water bodies and vegetatively reproduce by fragmentation (Ashton 1982, Lumpkin and Plucknet, 1982). From these results, there was no possible spread of the fern sexually. However the sample size was too small and further investigation would be needed to ascertain the spread of the fern if used in agriculture.

SH80 grown plants had significantly better yields than all treatments but SD20 grown plants had the lowest LAR indicating the best biomass allocation per available leaf

area. It would therefore be ideal to use the dry *Azolla* since less mass and volume is used but optimum yields are obtained under controlled conditions. The dry *Azolla* would be easier to work into the soil due a decrease in bulk. During the study period, it was observed that many insects were attracted to pots containing heated and fresh *Azolla* but not to the dry *Azolla* pots. This makes dry *Azolla* a better option since it provides less danger in spreading of diseases especially in the field where crops are exposed to uncontrolled conditions.

Based on the above results it was decided to use dried *Azolla* in the main experiment. Only 20% and 80% biofertiliser rates were used in the follow up experiment due to the lack of significant differences in above ground biomass of wheat plants grown in the dried *Azolla* treatments.

CHAPTER 3: COMPARING WHEAT GROWTH IN SAND AND TOPSOIL

After the preliminary experiment, a more detailed experiment was designed to address the questions that had arisen during the preliminary experiments and to confirm the results obtained with a greater level of confidence. In this experiment, the two growth

media were: sand and topsoil. Inorganic fertiliser and dry *Azolla* biofertiliser were mixed with sand and topsoil to produce different treatments. The number of pot replicates was increased from two to four for each harvest to increase the sample size. Plant productivity versus growth medium nutrient status was assessed by measuring various plant growth parameters as discussed in the following sections. The following specific questions were expanded upon with reference to the preliminary study results.

I) Does increasing the amount of dried *A. filiculoides* applied per volume of sand or soil from 20% to 80% improve the productivity of the wheat plants?
ii) Does the use of *A. filiculoides* as a fertiliser result in better wheat yields in acid washed sand in comparison to its application to field topsoil?
iii) How does the *A. filiculoides* biofertiliser in sand or soil compare to using inorganic fertiliser in either growth medium?
iv) What is the effect of the biofertiliser on the soil water and nutrients status at the different biofertiliser rates in both treatments?
v) In view of the fact that the fern is a weed, experiments to address the question of sporulation from the dried *A. filiculoides* as carried out in the preliminary study were repeated.

3.1 Materials and Methods

3.1.1 Growth medium

Table 18. Analytical results for topsoil texture, nutrient and micro nutrients. Analysis

done using Ambic-2 extraction method (Van der Merwe *et al.*, 1984). Data are means of three replicates. Soil was classified as Sandy loam.

Total sand	58.8±1.32%
Clay	17.53±0.58%
Silt	23.67±0.76%
Coarse sand	3.97±3.26%
Medium sand	8.9±3.31%
Fine sand	45.93±5.25%
Sand<0.1 mm	32.13±8.46%
Sand>0.1mm	26.7±9.76%
Nitrogen (Kjeldahl analysis)	0.13 %
Sample density	1.2±0.02 g ml^{-1}
Phosphorus	11.33±1.87 mg l^{-1} of soil
Potassium	171.33±2.25 mg l^{-1} of soil
Calcium	1283.67±14.11 mg l^{-1} of soil
Magnesium	131±23.72 mg l^{-1} of soil
Zinc	2.17±0.24 mg l^{-1} of soil
pH	4.13±0.03

Investigations were carried out on the effect of dried *A. filiculoides* as a biofertiliser of wheat (*Triticum aestivum* L. cv. Adam Tas). The same procedure for the *A. filiculoides* and sand growth medium was followed as in section 2.1.1, while topsoil was collected from a farm in Grahamstown. Topsoil texture and fertility analysis was done by Dohne Analytical services (Stutterheim), except for the total percentage N which was done by Matrolab chemical laboratory services(Brackenfell). Table 18 shows the results of the soil analysis.

3.1.2 Treatment

The harvested *A. filiculoides* was sun-dried and air-dried before application to the sand and topsoil at 20% and 80% (volume/volume) per pot. The amount of inorganic fertiliser (N: P: K 2:3:2, 50), used in the Grahamstown area was added as a treatment to both sand and topsoil at 30 kg N ha^{-1} (0.6594 g per pot) as recommended by an agricultural extension officer, Department of Agriculture. Controls of pure topsoil and sand were set up. Each treatment was replicated 16 times. The growth medium mixtures in the pots were left for one week in the greenhouse before planting. The treatments were as follows; Sand control (SC), Sand and 20% *Azolla* (S20), Sand & 80% *Azolla* (S80), Sand and NPK (SNPK), Topsoil control (TC), Topsoil and 20% *Azolla* (T20), Topsoil and 80% *Azolla* (T80). Topsoil & NPK (TNPK) The total number of pots was 128.

Split pot experiment

A split pot experiment was also set up. In contrast to the preliminary split pot experiment, the pots used in this experiment were cylindrical with a diameter of 10.8 cm. Each of the pots was partitioned into two with a perspex plane, 21 cm long. The perspex plane was fitted into a piece of circular wood (less than 2cm thick). This was then fitted into the bottom of the pot This was an improvement design from the preliminary experiment as the greater depth above the partition and enabled the roots to spread to both sides more easily. The control had pure acid washed sand on both sides of the partition. There were two treatments. In the first, 80% dried *A. filiculoides* with sand was placed in one side and pure acid washed sand was placed in the other side of the split pot. In the second, 80% *Azolla* was added to both sides of the split pot. Above the partition, each pot was evenly topped with pure acid washed sand to a height of four cm. Each treatment was replicated three times.

3.1.3 Plant material

Plant material was obtained and planted as in section 2.1.2. For the split pot experiment, the seeds were planted in the pure acid washed sand above the partition and in line with it. One week after germination, the plants were culled down to three per pot.

3.1.4 Growth conditions

All conditions were as described in section 2.1.3. At 36 days, a pesticide (Malasol; Efekto) was used to kill aphids. The plants were sprayed for red spider mite at 47 days after germination.

3.1.5 Plant harvest and measurements

The effects of the *A. filiculoides* in supplying nutrients were observed and recorded. Five plants were maintained per pot and four pots of each treatment were harvested at 25, 50, 75 and 128 days. The growth parameters found to be more sensitive from the preliminary experiment were measured during the final study. These were as follows: the total above ground dry biomass (leaves, leaf sheaths, kernel and grain) was measured. The inflorescence dry weight was measured separately from the straw (culms, leaves and leaf sheaths). The spikelet numbers, green (area of green leaves at time of measurement) and cumulative (area of both the green and dry leaves) leaf areas, Specific Leaf Area (leaf area per leaf weight) and RGR were also recorded. In addition, both above ground and below ground biomass were recorded at the 128 day harvest. Plants were oven dried at 65°C for 48 hours. From the oven, plants were placed in a dessicator before measuring the dry weights. R:S ratios were measured as dry root weight divided by the weight of the above ground biomass (Gomez-Macpherson *et al*., 1998a, Beadle, 1993).

In the split pot experiment, the partition was carefully removed and roots from each side separated from the soil, dried and weighed.

3.1.6 Soil water status

The effect of the application of *Azolla* on the soil water status in both sand and topsoil was investigated. After each of the first three harvests of the above ground biomass, these pots were watered once and left to drain for a period of 24 hours. Unlike in the preliminary experiment, the soil was not removed from the original pots. The pots were weighed daily using a 20 kg electronic scale (model FAT-12, NAGATA) for 22 days in order to measure the percentage water content for each treatment. Soil weights and soil water content were calculated as in section 2.1.5. After 22 days, there was no observable weight change and this was taken as the final weight.

3.1.7 Viability of spores

A small portion of each sand or soil-*Azolla* mixture was placed in Petri in the greenhouse for 2-3 months to allow viable spores to germinate after every harvesting of the wheat plants. The growth medium was kept wet throughout this experiment.

3.1.8 Data analysis

Data were analysed as in section 2.1.7.

3.2 Results

3.2.1 Productivity

3.2.1.1 Plant biomass

Sand treatments

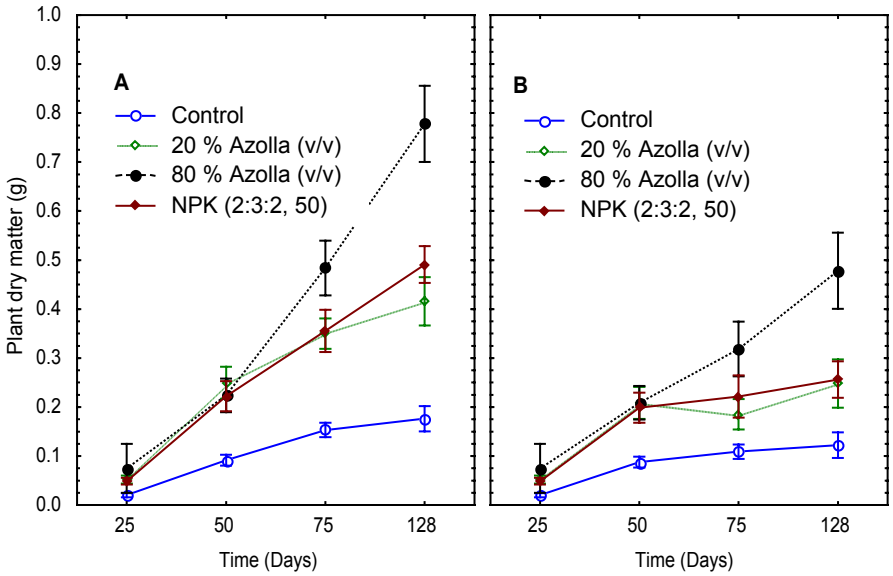

Fig. 6. Relationship between the total above ground plant dry matter (A) and dry straw (B) over time (days after emergence) in sand at different fertilisation rates with *Azolla* (v/v) and inorganic fertiliser. Data are means (n=20) with SE.

The response of total above ground dry matter (inflorescence, leaves and culms) and straw (culms, leaf sheaths and blades) of wheat plants grown in sand under different fertiliser (*Azolla* and inorganic) treatments was plotted against time (Fig. 6A & B).

Table 19. ANOVA results showing the effect of the sand treatments on the above ground and straw dry matter of wheat. Values are means of 20 plants at each harvest. Means followed by the same letters belong to homogeneous groups at a critical p-level of 0.05.

Day	Treatment	Above ground dry matter (g plant^{-1})	Straw dry matter (g plant^{-1})
25	SC	0.02 a	0.02 a
	S20	0.05 a	0.05 a
	S80	0.07 a	0.07 a
	SNPK	0.05 a	0.05 a
50	SC	0.09 a	0.09 a
	S20	0.25 b	0.21 b
	S80	0.22 b	0.21 b
	SNPK	0.22 b	0.20 b
75	SC	0.15 a	0.11 a
	S20	0.35 b	0.19 b
	S80	0.48 c	0.32 c
	SNPK	0.36 b	0.22 b
128	SC	0.18 a	0.12 a
	S20	0.42 b	0.25 b
	S80	0.78 c	0.48 c
	SNPK	0.49 b	0.26 b

At day 25 (Fig. 6A, Table 19), the total plant above ground dry biomass harvested from S20, S80 and SNPK pots showed no significant difference from the control. At day 50, there was a significant difference between all the fertilised treatments and the SC plants but not within the fertilizer treatments (Fig. 6A, Table 19). At day 75, the S80 grown plants had a significantly greater total above ground dry weight than S20 and SNPK plants which were significantly higher than the SC treated plants (Fig. 6A, Table 19). Thus, the S80 treatment outperformed the inorganic fertilizer (Fig. 6A). Between the treatments S20 and SNPK, no significant differences in dry matter were found over time (Fig. 6A). The S20 treatment, therefore improved the nutrient status of sand to the same level as the SNPK treatment. At day 128, differences between dry biomass of S80 and SNPK fertilised plants were significant with 342% and 179 % increase in comparison to SC grown plants respectively. There was no significant difference between the dry biomass of S20 and SNPK plants at the last harvest.

The response of straw dry matter (Fig. 6B, Table 19) shows similar significant trends to that of the total above ground biomass (Fig. 6A, Table 19). At day 50, all plants

harvested from fertilised treatments had significantly more straw than SC plants. At the 75 day harvest, the straw harvested from the fertilised treatments was significantly greater than that from the SC treatment. The straw harvested from the S80 treatment was significantly higher than the straw from S20 and SNPK grown plants. At the 128 day harvest, the S80 treatment showed a much higher difference in straw compared to the other fertilised treatments (Fig. 6B, Table 19). The S20 and SNPK grown plant straw was significantly greater than the straw of SC grown plants at day 128.

Although the dry plant matter and straw show the same trends in response to the different fertilisers, figures 6A and 6B showed that leaves, culms and grain contributed to increased biomass. The first two harvests showed no significant difference between the total above ground biomass and the dry straw matter. The third and fourth harvest were significantly different. Starting with day 75, there was hardly any increase in leaves and stems but a big increase in the inflorescence dry weights within each treatment. The S80 had the highest significant increases throughout the growth period (Fig. 6, Table 17) with the most biomass accumulation during the last three harvests. Biomass accumulation by the S80 plants was constant throughout the growth period.

The trend for S80 plants is different from S20 and SNPK plants (Fig. 6A) despite the fact that S20 plants flowered at 49 days after emergence while the S80 and SNPK had flowered at 58 days (Table 20). Earlier flowering did not have an effect on the accumulation of biomass within the fertilised sand treatments. The difference in flowering between the S20 and the fertilised sand treatments may be a result in difference in nutrient release by the differing quantities of *Azolla* present in soil.

Table 20. Day at which anthesis was observed for each treatment.

	Sand				Topsoil			
Treatment	**SC**	**S20**	**S80**	**SNPK**	**TC**	**T20**	**T80**	**TNPK**
Anthesis	58	49	58	58	47	46	46	46

(days)								

Topsoil treatments

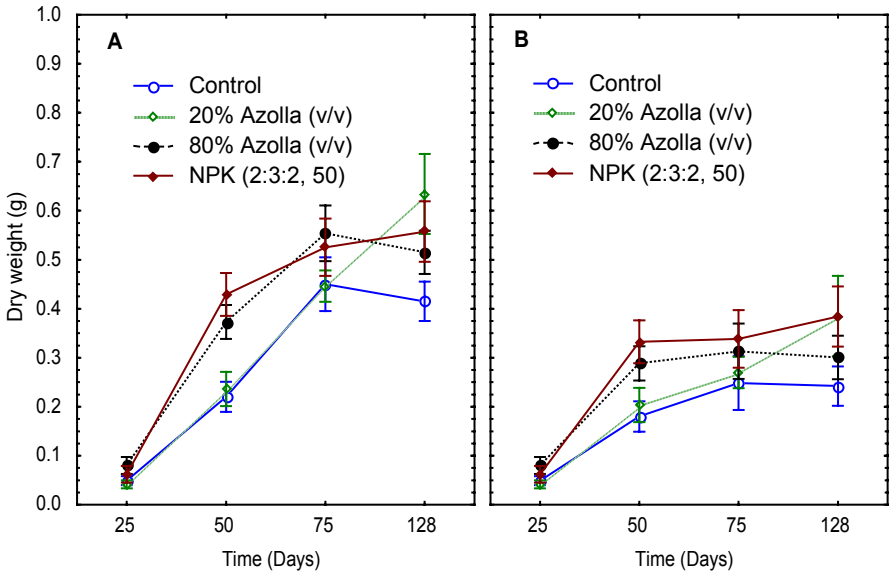

Fig. 7. Total above ground plant dry matter (A) and dry straw (B) in topsoil at different fertilisation rates with *Azolla* (v/v) and inorganic fertiliser for four harvests. Data are means (n=20) with SE.

Figures 7A and 7B show plots of plant total above ground and straw dry matter respectively, under different fertiliser regimes in topsoil over time.

In figure 7A, at day 50, the dry above ground biomass of plants grown in the TC pots was not significantly different from that of plants grown T20 pots. Both the T80 and TNPK grown plants had significantly greater biomass than the plants harvested from TC and T20 treatments (Table 21). Observations at the third harvest (75 day) showed that all plants from fertilised treatments did not have significantly higher dry matter than the control plants (Fig. 7A, Table 21). At the last harvest, the T20 treatment had the best effect in terms of plant dry matter but this was not significantly different from the rest of the fertilised plants, which were still significantly higher than the control. This suggests that an increase (20% to 80%) of *Azolla* has no effect in topsoil. The

slight decrease in the control and the T80 may be a result of using plants in different pots harvested at random, and poor grain filling, or as a result of a more severe red spider attack than on the other treatments. Therefore under ideal conditions, plants grown in TC and T80 might have increased in dry matter till day 128 to show a trend similar to TNPK plants, since topsoil appears to have had sufficient nutrients for above ground biomass accumulation (Fig. 7A).

In figure 7B, the dry straw matter shows the same trends as the total dry above ground matter within all the treatments. At the 50 day harvest, the T80 and TNPK showed a significantly higher difference in dry straw weight to the control. At day 75, T80 and TNPK grown plants had higher straw weight than TC plants (Fig. 7B). However at day 128, both the T20 and TNPK plants showed the highest response which is significantly different from the control (Table 21). There were no significant differences between the fertilised treatments at this harvest. Between harvests, the only significant difference in straw was at day 50.

The biggest contribution to the dry matter by the straw was during the first 50 days and thereafter, dry matter increase was contributed by the inflorescence (Fig. 7). The TC and T80 had significant total above ground dry matter increases over time until 75 days, however the T20 plants show a linear trend throughout the harvest. Plants in the lowest biofertilisation rate showed the highest biomass contribution by the inflorescence at day 128, but it was not significantly different from the other fertiliser regimes.

Table 21. ANOVA results showing the effect of the topsoil treatments on the above ground and straw dry matter of wheat. Values are means of 20 plants. Means followed by the same letters belong to homogeneous groups at a p-level of 0.05.

Day	Treatment	Above ground plant dry matter (g plant^{-1})	Straw dry matter (g plant^{-1})

25	TC	0.05 a	0.05 a
	T20	0.04 a	0.04 a
	T80	0.08 a	0.08 a
	TNPK	0.06 a	0.06 a
50	TC	0.22 a	0.18 a
	T20	0.24 a	0.20 a
	T80	0.37 b	0.29 b
	TNPK	0.43 b	0.33 b
75	TC	0.45 a	0.25 a
	T20	0.45 a	0.27 a
	T80	0.55 a	0.31 a
	TNPK	0.53 a	0.34 a
128	TC	0.41 a	0.24 a
	T20	0.63 b	0.39 b
	T80	0.52 b	0.30 ab
	TNPK	0.56 b	0.38 b

The plants in T20, T80 and TNPK pots flowered at 46 days followed a day later by the TC (Table 20). Anthesis differed only by a day between the TC and the fertilised plants.

Sand treatments versus topsoil treatments

There was an expected difference in biomass accumulation over time between the two controls with the topsoil doing best (Figs. 6 & 7). At the final harvest, application of 20% *Azolla* performed significantly better in topsoil than in sand. Therefore, plants grown in T20 had more nutrients available for growth than the S20 grown plants. The 80% *Azolla* performed best in the sand while the inorganic fertiliser, though better in topsoil, showed no significant difference from the dry matter response in sand. The addition of inorganic fertiliser and 20 % *Azolla* in sand gave the same results as plants that were grown in the topsoil control. The soil appears to be sufficiently high in nutrients to mask the effects of fertilisation at the 80% *Azolla* fertilisation rate.

In both soil and sand, biomass accumulation during the first 50 days was mainly in the form of stems and leaves. After day 50, the inflorescence mass contributed to dry matter content of all plants. There is a significant difference in the time of flowering in topsoil and sand (Table 20). Using a one way ANOVA, the plants in the soil flowered at a significantly earlier date than those in sand except in the S20 treatment. Macro nutrients are important for vegetative growth while the trace elements such as boron are needed for flowering. The availability of trace elements such as boron might have resulted in the differences in flowering.

3.2.1.2 Spikelet and grain relationships

The bar graphs in Figure 8 show the effect of sand (A-D) and topsoil (E-H) under different fertiliser regimes on the spikelet number and grain number of spring wheat plants. Each spikelet contained eight florets which either aborted or filled with grain.

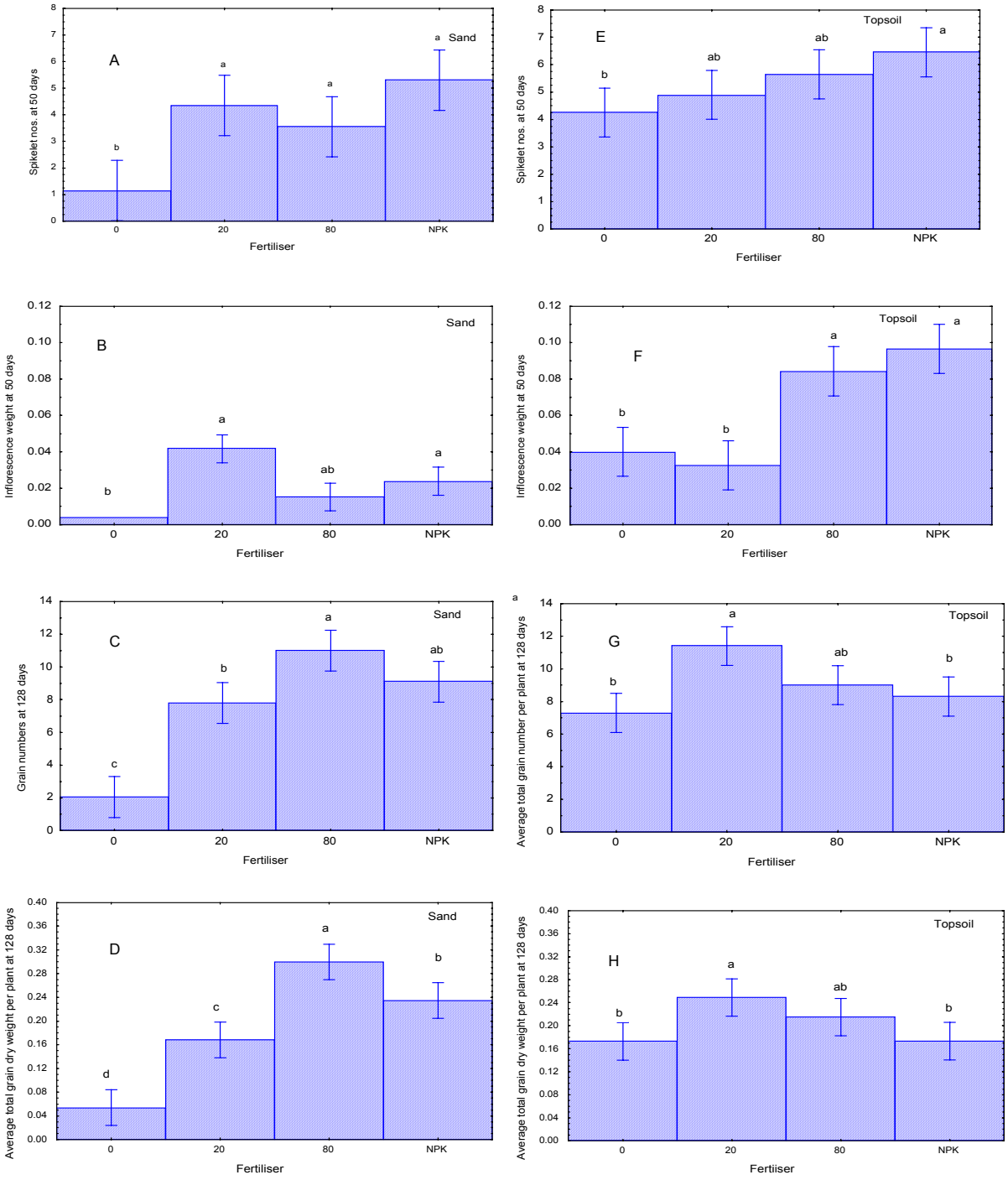

Fig. 8. Spikelet and inflorescence weights at the 50 day harvest, grain numbers and grain weights at the 128 day harvest in sand and topsoil under different fertiliser regimes. Data are means of 10 plants with SE at a critical p-level of 0.05. Means followed by the same letters belong to homogeneous groups.

<u>Sand treatments</u>

In Figure 8A, spikelet numbers are shown for each fertiliser regime at the 50 day harvest. The plant spikelet numbers in the SC treatment are significantly lower than those in the fertilised treatments which showed no significant difference between them.

Figure 8B shows the total corresponding weights of the spikelets per plant at the 50 day harvest. The dry weights of the inflorescence harvested from S20 and SNPK pots were significantly higher than the dry inflorescence weight of plants harvested from SC (Fig. 8B). There was no significant difference in inflorescence dry weights between SC and S80 grown plants. The total spike weight per plant for S20 grown plants was significantly greater than that observed for plants harvested from S80 and SNPK pots. This may be attributed to the fact that the plants grown in S20 developed buds earlier and then flowered at 49 days while plants in all SC, S80 and SNPK treatments flowered at 58 days (Table 20). The S80 and SNPK did not have significantly different inflorescence dry weights.

Figure 8C shows the final grain numbers at the 128 day harvest of plants grown in sand and the fertiliser regimes. The numbers of grain in all the fertilised treatments were significantly greater than those harvested from SC pots. The S20 grown plants had significantly lower grain numbers than S80 grown plants. There was no significant difference between S20 and SNPK grain numbers per spike. S80 and SNPK grain numbers per plant were also not significantly different.

The corresponding total grain weights at 128 days (Fig. 8D) reflect the same trend as the grain numbers. The grain dry weights of plants harvested from the fertilised treatments were significantly greater than that of plants harvested from SC pots. S20 grown plants had significantly lower total grain weights than S80 and SNPK grown plants. S80 total grain weight per plant was significantly greater than that of plants harvested from SNPK pots.

When the numbers of the spikelets present at the 50 day harvest were compared to the grain numbers at day 128, the trends were similar (Figs. 8A &C). In both figures the SC numbers are significantly reduced in comparison to the fertilised treatments. Fertiliser and biofertiliser application to sand improves spikelet numbers per plant and results in greater grain numbers. However, at day 128, the S80 grown plants had the highest number of grains which was significantly greater than that of S20 plants. The inflorescence and the grain weights of plants harvested from the SC treatment were significantly less than those of all plants harvested from the fertilised treatments (Figs. 8B & D). Interestingly, at day 50, S20 plants had a significantly greater spike weights, but at day 128 they had the lowest spike weights in comparison to all the plants harvested from the fertiliser treatments.

Topsoil treatments

Figure 8E shows the number of spikelets in the various topsoil treatments at the 50 day harvest. There was no significant difference in spikelet numbers between the TC, T20 and T80 treatments (Fig. 8E). The spikelet numbers on TNPK grown plants were significantly greater than those of TC grown plants. For the fertilised treatments, there was no significant difference in spikelet numbers per plant.

The corresponding weights of the inflorescence in topsoil at day 50 are shown in figure 8F. The TC plant inflorescence weight is not significant from that of T20 plants but is significantly lower than T80 and TNPK grown plants. T20 plants had significantly lower spike weights per plant than T80 and TNPK plants. T80 spike weight per plant is not significant from TNPK spike weight per plant. The heavier spikes suggest that the plants grown in the highest fertiliser mixtures had invested much more in their reproductive structures than the TC and T20 plants. At day 50, the spikelet numbers are not different but the spike weights were increased at higher nutrient levels.
The number of grains observed at the last harvest are shown in a bar graph (Figure 8G). The number of grains in TC grown plants was significantly lower than that of plants harvested from T20 pots but not significantly different from that of T80 and

TNPK grown plants. T20 plants did not have significantly different grain numbers from T80 plants but had significantly higher grain numbers than TNPK plants. The highest fertiliser treatments showed no significant difference in grain numbers.

Figure 8H shows the grain dry weights harvested at day 128. This showed the same trend as the grain numbers. The plants harvested from TC pots had significantly lower grain weights than the plants harvested from T20 pots but not significant from those of plants grown in the highest fertilisation treatments. T20 grown plants did not have significantly different grain weight from T80 plants which had significantly greater grain weight than TNPK grown plants. The highest fertiliser regimes show no significant difference from each other. Although the spikes of T80 and TNPK grown plants were heavier than those of T20 grown plants at 50 days, their final grain weight at 128 days was not heavier than that of the T20 plants.

Sand treatments versus Topsoil treatments.

At the 50 day harvest, the spikelet numbers for the SC grown plants (Table 22) were significantly lower than the plants harvested from the topsoil treatments (Table 22). The spikelet numbers in the S20 plants were the same as those observed in T20 grown plants. There was no significant difference in spikelet numbers within the NPK fertilised sand treatments and all topsoil treatments.

Table 22. Spikelet and inflorescence weights at the 50 day harvest, grain numbers and grain weights at the 128 day harvest in sand and topsoil under different fertiliser regimes. Data are means of 10 plants with SE at a critical p-level of 0.05. Means followed by the same letters belong to homogeneous groups.

Fertiliser	Treatment	Spikelets No. spike^{-1} (50 days)	Inflorescence weight g plant^{-1} (50 days)	Caryopses No. spike^{-1} (128 days)	Grain weight g plant^{-1} (128 days)
0	Sand	1.15c	0.003 c	2.05 c	0.05 c
	Topsoil	4.25 ab	0.04 b	7.30 b	0.17 b
20	Sand	4.35 ab	0.04 b	7.80 b	0.17 b
	Topsoil	4.90 ab	0.03 bc	11.40 a	0.25 a
80	Sand	3.55 b	0.02 b	11.00 a	0.30 a
	Topsoil	5.65 ab	0.08 a	9.00 ab	0.21 b
NPK	Sand	5.30 ab	0.02 b	9.10 ab	0.23 a
	Topsoil	6.45 a	0.10 a	8.30 b	0.17b

The inflorescence dry weights in SC (Table 22) were significantly lower than all the spike weights of plants harvested from topsoil treatments except T20 (Table 22) at the 50 day harvest. At this harvest, spike weights per plant grown in the S20 treatment were not significantly different from the TC and T20 plants. However, plants grown in the highest fertiliser sand mixtures had significantly lower spike weights than plants from T80 and TNPK treatments.

At the 128 days, the grain numbers of plants harvested from the SC (Table 22) pots were significantly less than those of plants grown in the topsoil treatments (Table 22). S20 performed as well as TC but significantly lower than T20 which is not significantly different from S80. A comparison between S80 and T80 showed no significant difference in grain numbers. The SNPK and TNPK grown plants also had similar grain weights per plant. The numbers of grain in the fertilised sand treatments lie within the same range as those in all topsoil treatments.

The grain dry weights in SC (Table 22) were significantly lower than grain weights of plants harvested from all topsoil treatments (Table 22). Total grain harvested from S20 pots weighed less than grain harvested from T20 pots. The S80 average total grain weighed per plant is not significantly different from the T20. Within the 80% *Azolla* and NPK treatments, there is a significant difference between sand and topsoil.

3.2.1.3 Relative growth rate (RGR; g g^{-1} day^{-1})

Sand treatments

Figure 9 is a plot of the relative growth rate of the total above ground biomass (A) and the straw (B). Between the different sand treatments, there were no significant differences in the dry matter accumulation as shown by the RGR for both the vegetative and reproductive tissue over a specific sampling date interval. Differences in RGR were observable within each treatment over the first two sampling intervals.

The relative growth rate of the total above ground biomass (Fig. 9A) showed no significant differences between treatments over the first 50 days. Over the period of 50 to 75 days, the RGR of S80 grown plants was the highest but not significantly different from the other treatments. From day 75 to 128, there was no significant difference between the SC and the fertiliser treatments, although S80 grown plants still had the highest RGR. There was a steady decline over time in the RGR of the total above ground biomass.

Figure 9B shows the RGR of the total stem and leaf dry matter per plant. There were no significant differences between treatments during the 25-50 day interval. Over the 50 to 75 interval, the RGR of S80 was the highest but only significantly different from S20. Over the 75 to 128 day interval, there was no significant difference between treatments.

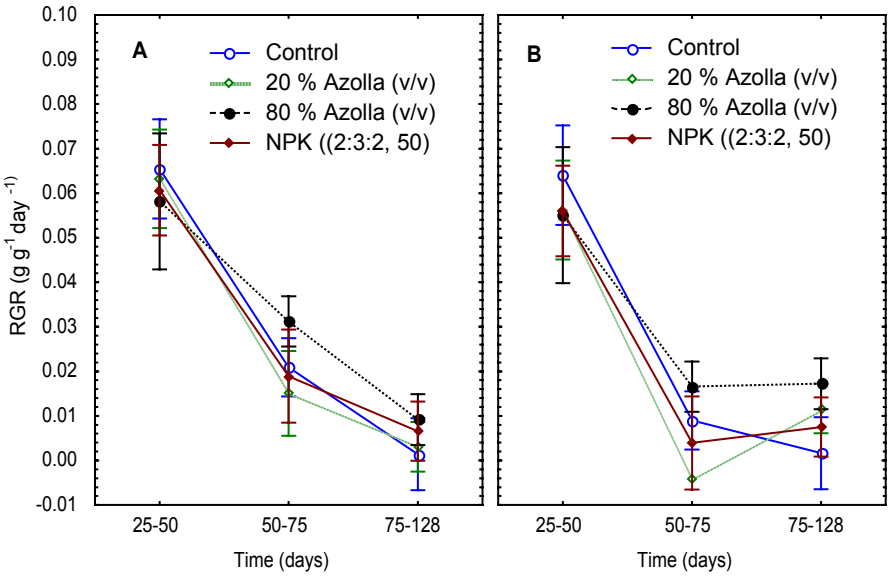

Fig. 9. Relative Growth Rates (RGR) of total above ground biomass (A) and straw (B; culms, leaf sheaths and blades) during the last three harvests in sand at different fertiliser rates with *Azolla* (v/v) and inorganic fertiliser. Data are means (n=20) with SE.

Both figures 9A and B show that the most rapid decline in RGR was between the first and second intervals and hardly any decline between the 2nd and 3rd sampling intervals. Most of the vegetative matter was produced in the first 50 days, during which the highest RGR values were observed. Thereafter, decline in RGR is noted as a result of grain production. There are significant differences within the treatments between the 25 to 50 and the 50 to 75 day intervals. The general lack of significant differences between the treatments in sand and topsoil at each time interval suggests that the addition of fertiliser had little effect on the RGR of the wheat plants.

<u>Topsoil treatments.</u>

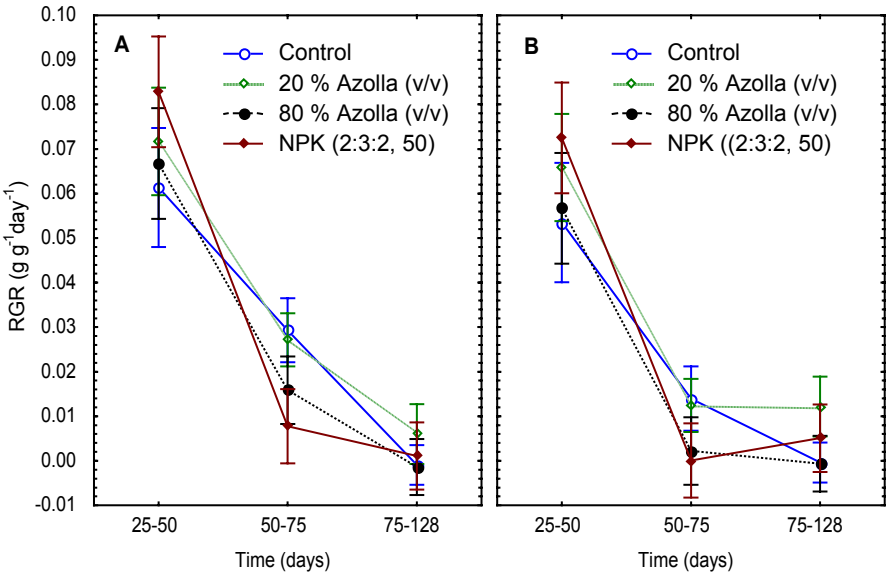

Fig. 10. Relative growth rates of total above ground biomass (A) and Straw (B; culms, leaf sheaths and blades) during the last three harvests in topsoil at different fertiliser rates with *Azolla* (v/v) and inorganic fertiliser. Data are means (n = 20) with SE.

In Figure 10, the RGR of the total above ground biomass (A) and the straw (B) are plotted against time. Plants in topsoil showed similar trends to plants grown in sand in that there were no significant differences between treatments over specific sampling intervals. However there was a general decline in RGR over time.

There was no significant difference in total above ground dry matter accumulation between treatments during the first 50 days (Fig. 10A). Over the 50 to 75 day interval the TNPK had the lowest RGR which was significantly lower than TC and T20. However, over the 75-128 day sampling interval there was no significant difference between the treatments.

The straw dry matter (Fig. 10B) showed no significant differences between the various treatments at each interval over time.

In Figure 10, over each time interval, there was no significant differences within each treatment between the total above ground (Fig. 10A) and the straw (Fig. 10B). The highest dry matter accumulation was over the 25 to 50 day interval which was significantly higher than the 50 to 75 day interval RGR for both total above ground biomass and straw (Figs. 10A & B).

Sand versus topsoil treatments.

The highest RGR was shown by the plants grown in topsoil (Fig. 10) The steeper decline between the first two time intervals in RGR shown by the plants in topsoil suggests that these plants were producing vegetative tissue faster and therefore attained maximum mass faster than those in sand (Fig. 10). Plants in all topsoil treatments show a sharp increase in above ground biomass (Figs. 6A & 7B) which is not significantly different from that of S80 (Figs. 1A & 1B) grown plants to day 75. Therefore the topsoil grown plants were bigger and grew faster than SC, S20 and SNPK plants. Although the RGR was higher in topsoil (Fig. 10) than in sand (Fig. 9) for all treatments at each time interval, there were no significant differences.

3.2.1.4 Green and cumulative leaf areas (cm)

Sand treatments

Figure 11 shows the leaf area response of plants grown in sand and the various fertiliser treatments. At three weeks after emergence, plants in fertilised sand treatments showed a notable difference in leaf areas from the control plants. The use of fertilisers in sand resulted in increased leaf areas per plant.

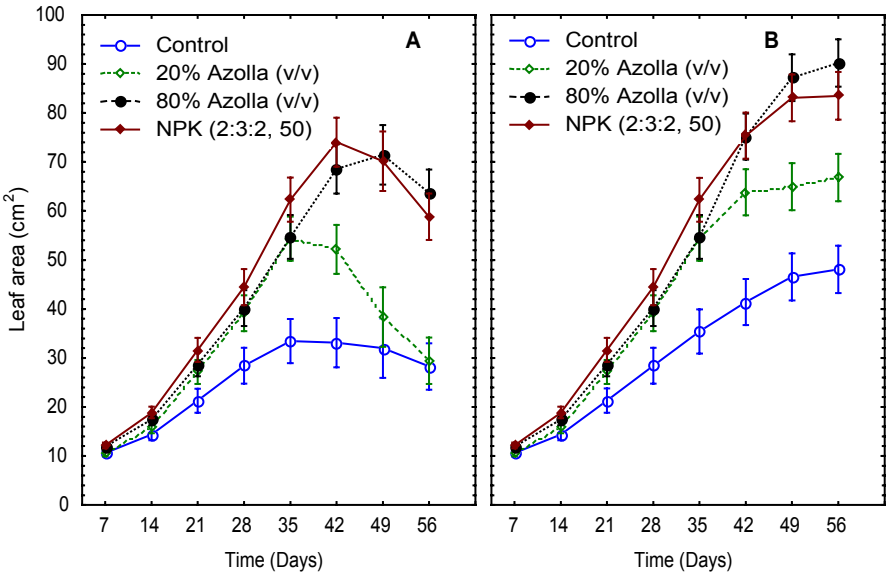

Fig. 11. Green (A) and cumulative (B) leaf areas and measured at one week intervals in sand at different fertilisation rates with *Azolla* (v/v) and inorganic fertiliser over time. Data are means (n = 20) with SE.

In figure 11A, the green leaf areas are plotted at one week intervals. The application of fertiliser resulted in no significant difference of green leaf areas between all treatments at days seven and 14 (Fig. 11A). Over the 21 to 42 day period, the SC treatment had significantly reduced green leaf areas in comparison to the fertilised treatments. Therefore, the application of fertiliser increases the green leaf areas of wheat plants grown in sand. Within the fertilised treatments, the highest green leaf area was attained in SNPK at 42 days which was a week later than the maximum green leaf area in S20 and a week earlier than that in S80 (Tables 22 & 23). There was a significant difference between maximum green leaf area of S20 and the other fertiliser rates. However there was no significant difference in green leaf areas between S80 and SNPK throughout the growth period. The average rate of decrease of green leaf areas is 5% for SC, 15% for S20, 11% for S80 and 10% for SNPK per week after attaining their maximum green leaf areas.

Figure 11B is data of the cumulative leaf area of a plant which includes both the dry

and green leaf areas at a specific time. Figure 11B showed that there was a significant difference between the cumulative leaf area of the SC and the fertilised treatments starting at 21 days. Starting at 42 days, S20 cumulative leaf areas were significantly lower than S80 and SNPK which are not significantly different. The highest cumulative leaf areas were at day 56 after the flag leaves were fully formed.

Table 23. Maximum green leaf areas (GLA) and cumulative leaf areas (CLA) of plants for all treatments. Data are means of 10 plants with SE.

Treatments	SC	S20	S80	SNPK	TC	T20	T80	TNPK
GLA cm^2	33.44	54.35	71.48	74.05	42.85	52.44	57.25	66.66
	±4.5	±4.5	±6.06	±5.01	±5.02	±5.01	±4.5	±4.5
CLA cm^2	48.07	66.81	90.05	83.50	49.82	58.96	66.33	72.09
	±4.85	±4.85	±4.84	±4.85	±4.84	±4.84	±4.84	±4.78

Within the SC and S20 treatments, there was a difference between the biggest green leaf area at 35 days (Fig. 11A, Table 23) and the significantly bigger cumulative leaf area at 56 days (Fig. 11B, Table 20). SNPK and S80 green leaf areas peaked at 42 days and 49 days respectively (Fig. 11A, Table 23) while the significantly higher cumulative leaf areas saturated at 56 days (Fig. 11B, Table 23).

Topsoil and topsoil fertiliser treatments

In Figure 12, the green (Fig. 12A) and cumulative (Fig. 12B) leaf areas of wheat grown in topsoil were plotted at one week intervals for a period of 7 to 56 days. There was no significant difference between treatments from seven to 21 days (Fig. 12A). At 28 days, there was no significant difference between TC and T20 but these were significantly lower than T80 and TNPK which were not significantly different from each other. At 35 days, T80 and TNPK attained maximum green leaf areas per plant while that of TC and T20 maximum green leaf areas were attained a week later (Fig. 12A,

Table 23 & 24). The biggest green leaf area of TC plants was not significantly different from T20 but was significantly lower than that of T80 and TNPK. The greatest green leaf areas of T20 were significantly lower than that in TNPK. The decline in green leaf areas was 23% in TC, 1% in T20, 21% in T80 and 28% in TNPK per week after attaining the maximum green leaf areas.

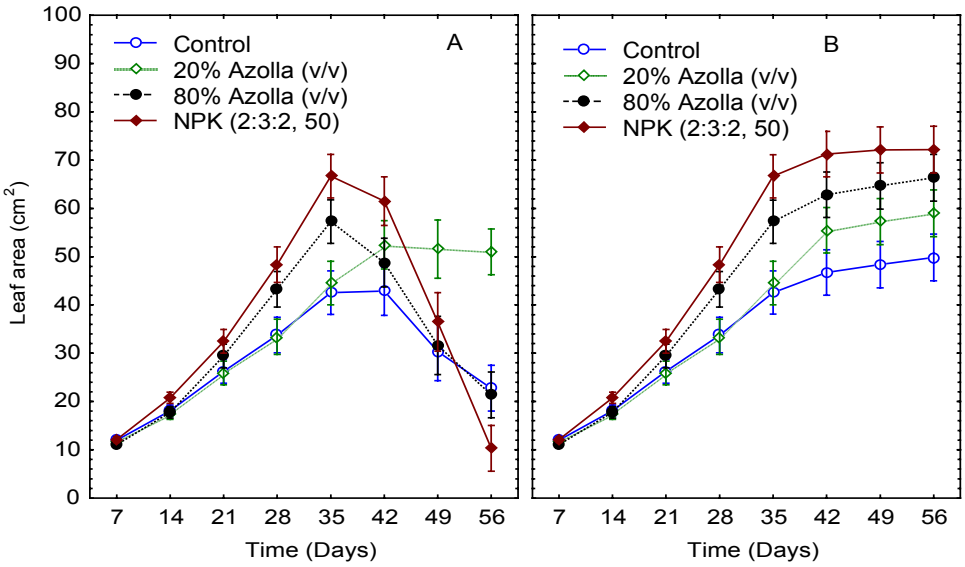

Fig. 12. Green (A) and Cumulative (B; dry and green) leaf areas measured at one week intervals in topsoil at different fertiliser rates with *Azolla* (v/v) and inorganic fertiliser over time. Data are means (n = 20) with SE.

In Figure 12B, the cumulative leaf areas showed no significant differences between treatments. For the period of 7-21 days, there were no significant differences observed between the topsoil treatments. At 28 and 35 days, TC and T20 were not significantly different but were significantly lower than T80 and TNPK. Starting from 42 days to 56 days, the cumulative leaf areas in TC were significantly lower than T80 and TNPK but not significantly different from T20. During this period T20 was not significantly different from T80 but was significantly lower than TNPK. The biggest

cumulative area was in TNPK at 42 days while the others peaked at 56 days.

Although the highest cumulative leaf areas are bigger than the highest green leaf areas, there is no significant difference between them within each treatment (Figs. 12A & B).

Sand versus topsoil treatments

Table 24. Maximum green leaf areas (GLA) peaks for all treatments.

Treatments	SC	S20	S80	SNPK	TC	T20	T80	TNPK
GLA (days)	35	35	48	42	42	42	35	35

The green leaf areas in SC and S20 peaked at 35 days while TC and T20 peak at 42 days, S80 peaked at 49 days while T80 peaked at 35 days and SNPK peaked at 42 while TNPK peaked at 35 days (Figs. 11 & 12, Table 24). Between topsoil and sand, green leaf areas were only significantly different within the controls and the highest biofertiliser treatment. The TC grown plants had higher green leaf areas than SC grown plants which is a result of presence of more nutrients in topsoil. T80 plants had reduced leaf areas in comparison to S80 plants. The T20 and S20 grown plants showed no significant difference. Significant differences between treatments were observed at 21 days in sand and at 28 days in topsoil. Except for T20, the rate of leaf senescence was faster in topsoil than in sand after maximum green leaf areas had been attained. There was no significant difference between the cumulative and green leaf areas in topsoil grown plants, unlike plants grown in sand *Azolla* treatments. Within treatments, there were significantly higher cumulative leaf areas in sand than in topsoil for the 80% *Azolla* and inorganic fertiliser mixtures.

3.2.1.5 Specific Leaf Areas (SLA; $cm^2\ g^{-1}$)

Response of specific leaf areas in sand and topsoil treatments

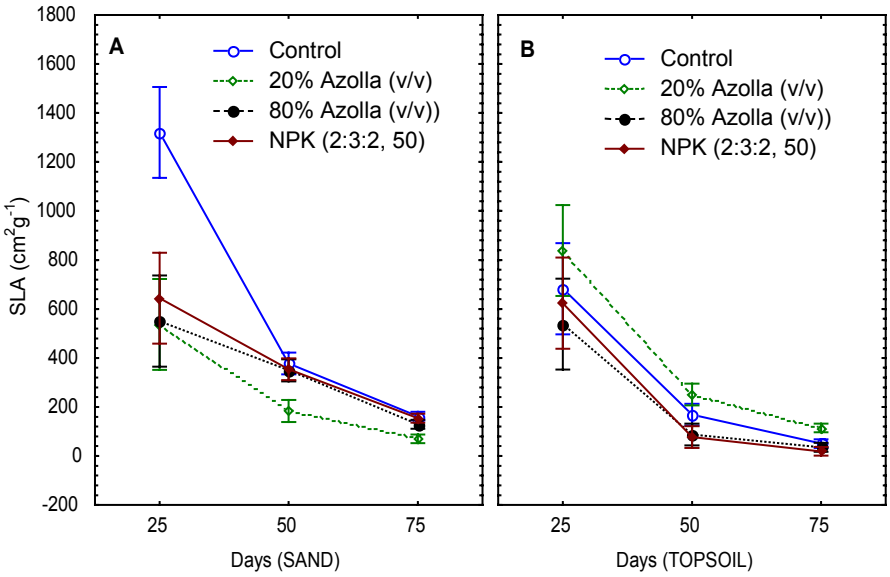

Fig. 13. Specific Leaf Areas (SLA) during the last three harvests in sand (A) and topsoil (B) at different fertiliser rates with *Azolla* (v/v) and inorganic fertiliser over time. Data are means (n = 20) with SE.

Figure 13 shows a plot of specific leaf areas of plants harvested from sand (A) and topsoil (B) in response to different fertiliser treatments. The SC SLA was significantly higher (Fig. 13A, Table 24) than the fertiliser treatments. However at days 50 and 75, S20 was significantly lower than the other treatments which showed no difference between them. Therefore the leaves of S80 and SNPK grown plants have bigger leaf areas without an increase in carbon accumulation, which would result in greater leaf mass. The S20 grown plants have a lower SLA which suggests a much more efficient carbon accumulation than the other treatments. There is a significant decrease over time as leaves get heavier relative to their leaf area.

Table 25. ANOVA results showing the effect of the sand and topsoil treatments on the specific leaf area (SLA) of wheat. Values are means of 20 plants. Means followed by the same letters belong to homogeneous groups at a p-level of 0.05.

Day	Treatment	SLA ($cm^2\ g^{-1}$) $plant^{-1}$ in sand	SLA ($cm^2\ g^{-1}$) $plant^{-1}$ in topsoil
25	Control	1320.60 a	682.80 a
	20% *Azolla*	536.96 b	838.61 a
	80% *Azolla*	550.58 b	538.04 a
	NPK	644.07 b	623.77 a
50	Control	377.61 a	168.72 ab
	20% *Azolla*	183.50 b	251.28 a
	80% *Azolla*	353.68 a	87.70 b
	NPK	349.68 a	77.42 b
75	Control	162.64 a	50.51 a
	20% *Azolla*	69.97 b	114.27 b
	80% *Azolla*	129.12 a	35.67 a
	NPK	154.08 a	18.87 a

Figure 13B showed no significant differences between the treatments at day 25. At day 50, the T20 grown plants had significantly higher SLA than T80 and TNPK grown plants (Table 25). The SLA of T80 and TNPK plants was not different form that of TC grown plants. This suggests that nutrient supply to T80 and TNPK grown plants had no effect on carbon allocation to leaves of plants grown in topsoil.

At 25 days, SLA of SC grown plants was significantly higher than SLA of plants grown in all topsoil mixtures. However, the plants grown in fertiliser mixtures in sand are not significantly different from all the plants grown in topsoil mixtures. At 50 days, S20 is significantly lower than T20 but not different from TC, T80 and TNPK. The SLA of SNPK and S80 grown plants was not different from that of T20 grown plants. At 75 days, S20 is not different from TC, T80 and TNPK but is significantly lower than T20 which is not different from SC, S80 and SNPK.

3.2.1.6 Root to shoot ratios (R:S)

Root to shoot ratios in sand treatments

Table 26. Results of plant biomass harvested at 128 days. Data are means of 20

plants with SE in parentheses. Values followed by the same letters belong to homogeneous groups at a critical p-level of 0.05.

Treatments	Plant biomass		
	Root (g plant^{-1})	Shoot (g plant^{-1})	Root: Shoot
	F= 5.7547	F=101.9355	F=12.2268
	p=0.00529	p=0 .00012	p=0.00004
Sand	0.0466	0.1762	0.3718
	(± 0.01153) ab	(± 0.0334) c	(± 0.2263) a
Sand & NPK	0.0576	0.4909	0.1046
	(± 0.0150) b	(± 0.0501) b	(± 0.0412) b
Sand &	0.0758	0.7779	0.1216
80% *Azolla*	(± 0.0163) a	(± 0.0830) a	(± 0.0252) b

At day 128, the R:S ratios between the S80 and the SNPK are not significant but these two are significantly lower than the control (Table 26). This suggests that plants in pure sand invested more in their underground biomass in an effort to acquire nutrients for their above ground biomass. The mean is higher in the S80 than the SNPK treatment. The root to shoot ratios of the topsoil plants were not calculated due to difficulty in separating the *Azolla* from the roots at harvest.

3.2.2 Percentage soil moisture content

Effect of biofertiliser on water content in sand and topsoil treatments

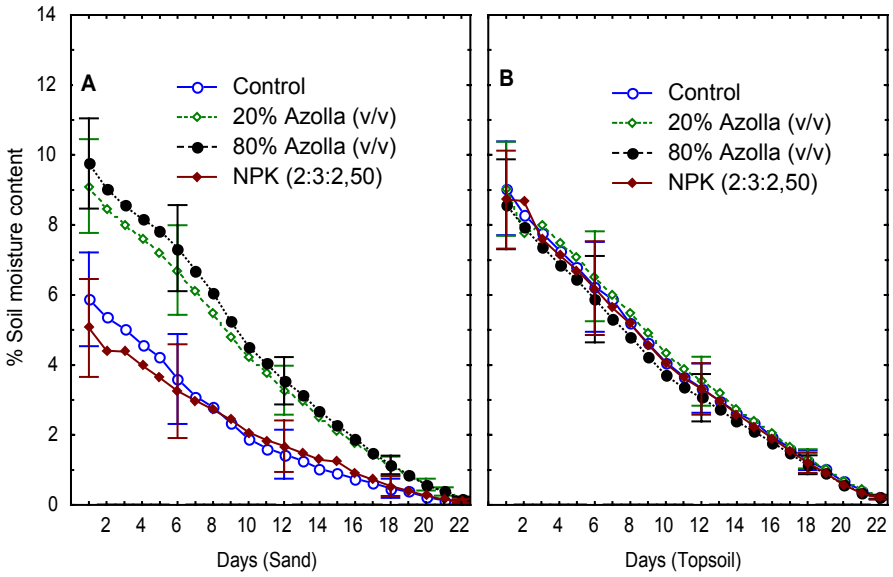

Fig. 14. Percentage soil moisture content measured at one day intervals in sand (A) and topsoil (B) at different fertilisation rates with *Azolla* (v/v) and inorganic fertiliser over time. Data are means (n = 20) with SE.

The percentage moisture content in the different fertiliser treatments within sand and topsoil was plotted at one day intervals over a period of three weeks (Fig. 14A & B). Figure 14A, there was no significant difference between the SC and SNPK treatments. There was a significant effect of biofertiliser (S20 and S80) in sand for the first 14 days (Fig. 14). The percentage moisture content in S20 and S80 was significantly higher than in SC and SNPK. Although, the S80 moisture content was higher than that in SC during the first 11 days, no significant differences were observed. The significant difference between the biofertiliser treatments and the non-biofertiliser treatments suggests that the water holding capacity in sand is improved by the addition of biofertiliser to sand.

Figure 14B shows the water holding capacity of topsoil treatments. The soil moisture content in topsoil was not affected by the addition and increase of fertilisation (Fig. 14B). All treatments were not significantly different form each other.

SC and SNPK moisture content was significantly lower than all topsoil treatments (Figs. 14A & B). There were no differences in the S20, S80 treatments and the soil mixtures which indicates that biofertilisation makes the sand as good as the soil in holding water, but has no effect in the soil.

3.2.3 Sporulation

Frond appearance in sand and topsoil treatments

The first frond to appear was in sand taken from the S20 treatments. It appeared after a period of 78 days in the Petri dish. The frond could only have germinated from a spore that was well protected in the mat of *Azolla* that had been dried prior to use as a biofertiliser. The sporeling grew until it filled an area of more than half of the Petri dish.

The next frond grew from the T20. It took 96 days for the a frond to appear, which was 28 days later than the spore inn sand. Unlike the sporeling in sand, this one remained as a single frond and had a lifespan of 20 days.
Although the sample size is very small, the results show that spores are able to germinate in both sand and topsoil. They were resistant to being air dried at temperatures above 25°C and being thoroughly mixed and buried in topsoil and sand after 25 days as a fertiliser.

3.2.4 Split pot experiment

Table 27. Means of the weights of roots collected from each side of the split pot. Data are means (n=3).

Treatment	S80 SC	S80 S80	SC SC

means	0.04963	0.0047	0.358	0.0239	0.0335	0.0391
p	0.0173*		0.5161^{ns}		0.8108^{ns}	

The average dry weight of roots collected from the split pot that contained S80 treatment on either side showed no significant differences (Table 27). Results from the split pot that contained S80 on one side and SC treatment on the other side showed that there were more roots recovered from the S80 side and this was significantly greater than the weight of the roots collected from the SC side. There was no significant difference observed in the root weights collected from either side of the control. More root ramification was observed in pots with *Azolla*. This may have led to poor recovery of roots from the biofertilised growth media. The above ground biomass and grain weight was the same for all plants in the fertilised split pots but a difference of more than 80% from the control which was observed in the non split pots.

3.2.5 Summary of performance of different treatments

Table 28 shows that the best above ground biomass at day 128 was effected by the application of 80% *Azolla* to sand and 20% *Azolla* to topsoil. S80 appears to have as much nutrients as T20 and TNPK, as indicated by straw biomass accumulation. All topsoil and S20 grown plants flowered earlier than the SC, S80 and SNPK plants. This maybe due to earlier availability of nutrients necessary for floral initiation by the plant. Although anthesis occurred later in S80 and SNPK plants, at 50 days these plants had the same number of spikelets as the topsoil and S20 grown plants. Like the above ground biomass, the S80 and T20 grown plants had the best grain weights which would suggest that in these treatments, nutrients were available for both straw and grain production. Despite the differences in above ground biomass, all plants had similar RGR values suggesting that the bigger plants grew faster than plants with lower final dry weights. The maximum green leaf areas of S80 and TNPK grown plants are similar to results in the above ground biomass. S20 treatment produced the best SLA in the wheat plants. However, the difference in SLA between treatments was small indicating that treatment had little effect on the structural matter accumulation in

leaves. The R:S ratios of SNPK and S80 plants indicate that most biomass was allocated above ground than to below ground plant matter.

Table 28. Summary of the response of the growth parameters to various treatments.

Growth parameter	Treatment with best response	Homogenous group members	Treatment with poorest response	Homogenous group members
Total above ground biomass	S80	T20	SC	NONE
Straw biomass	S80	T20, TNPK	SC	NONE
Anthesis	T20, T80, TNPK, TC	S20	SC	S80, SNPK
Spikelet nos.	SNPK	S20, S80, TC, T20, T80,TNPK	SC	NONE
Grain weights	S80	T20	SC	NONE
RGR	ALL	ALL	ALL	ALL
GLA	S80	SNPK, TNPK	SC	NONE
CLA	S80	SNPK, TNPK	SC	TC
SLA (25 days)	S20	ALL	SC	NONE
Root: shoot	SNPK	S80	SC	NONE

Most growth parameters show that the S80 treatment was the best performer (Table 28). This was followed by the T20 treatment. All growth parameters of plants harvested from the SC treatment indicated that the control had the poorest effect on plant productivity. This shows that sand had the poorest nutrient levels available for plant uptake.

CHAPTER 4: DISCUSSION

Most studies on the use of *Azolla* as a green manure have been carried out on rice under flooded conditions. In lowland rice cropping, Ito and Watanabe (1985) reported that the incorporation of *Azolla* in soil reduces the loss of N from the biofertiliser and enhances the availability of nutrients to plants. Rice grain and straw yields were improved by the addition of *Azolla* (Kannaiyan, 1993,Kondo, *et al*., 1989, Ventura &

Watanabe, 1993, Singh & Singh, 1990). Several studies focused on the residual effect of the *Azolla* on wheat. The use of *Azolla* was found to have beneficial effects on grain and straw yields of rice with residual positive effect on the following wheat crop yields (Mahapatra & Sharma, 1989, Kolhe & Mittra, 1990) Fewer studies have been done on the direct effects of *Azolla* on wheat. It has been found that the application of fresh fronds of *A. pinnata* harvested from a nearby pond increased the grain and straw yields of wheat (Prasad &Ram, 1981, Ram & Prasad, 1982, 1983). Pot grown *A. pinnata* that had been enriched with phosphate fertiliser was incorporated into soil (3 t ha^{-1}) to investigate the response of wheat. The fresh fronds had better effect on wheat grain yield than the dry biomass, although both hardly affected the straw yields (Marwaha *et al*., 1992). But in this study, at the 20% and 80% biofertiliser rate, both total above ground biomass (Fig. 2, Table 5) and grain weight (Table 7) per plant were significantly greater in the dry treatments than in the fresh treatments.

In most of the studies cited, emphasis was placed on the final grain and straw yields. However in this study, a wider range of growth parameters were measured at regular intervals throughout the growth period of the wheat plants. Furthermore, no previous study has taken the direct application of dried A. *filiculoides* and its effect on wheat as the first crop into account. This is important since the levels of N released may vary with *Azolla* species and their environment (Lumpkin & Plucknett, 1982). In addition, these studies have been carried out in Asia and not in South Africa where *A. filiculoides* has been found to grow profusely on water bodies and is a weed (Hill, 1999). At the commencement of this study, biological control of *A. filiculoides* had not been fully investigated and found successful. It was therefore hypothesised that the use of the weed as a biofertiliser would be a contribution to weed control on small water bodies such as farm dams and an effective use of a natural resource in South Africa.

4.1 Nutrients and plant biomass

In a study on the release of N from nitrogenous plant materials, Muller and Sundman

(1988) found that there was a high retention of plant derived N and relatively small loss of this N from the soil and that their value as supply to subsequent crops was long-term. This agrees with Ventura *et al*., (1987) who found that the addition of *A. microphylla* significantly increased rice grain yields only after the second application of *Azolla* at the second crop harvest. However, short term effects from the addition of the *Azolla* fern were observed for all treatments in both this preliminary and final study. This may be explained by earlier findings (Kannaiyan, 1993, Marwaha *et al*., 1992, Lumpkin and Plucknett, 1982) that the addition of *Azolla* increased nutrient availability as well as narrowing the C/N ratio in the soil that leads to faster decomposition of organic matter. In preliminary experiments of this study, the plants grown on treatments SF80, SD50 and SH80 produced the most dry matter (Fig. 2, Table 5). This would suggest that the pre-treatment and amount of the fern present in the soil affects nutrient release and availability in the soil. Furthermore, the heated and dried *Azolla* effected significantly greater above ground biomass (Tables 2 & 5) than the fresh *Azolla*. This difference in above ground biomass may be a result of nutrient availability due to the *Azolla* incorporated into soil as found by Watanabe and Ramirez, 1990 and Watanabe *et al*., 1991. The beneficial results showed by application of dried *Azolla* grown plants are especially relevant as air drying reduces the bulk of the *Azolla* and makes it easier to transport. The preliminary study agrees with previous studies in that the addition of *A. filiculoides* to acid washed sand improved the growth of wheat plants since the response of wheat in all the three treatments (Table 17) had significantly greater growth parameters than the pure sand control. This was confirmed in the main study. The sand was poor in nutrients due to acid washing and therefore good response of plants can be attributed to the organic matter and nutrients released into sand by the decomposition of the *Azolla* fern (Ventura & Watanabe, 1993, Rengel *et al*., 1999). From this study, the addition of nitrogenous plant material to nutrient poor sandy soils resulted in increase yields over short term conditions and therefore one would expect beneficial long term results due to residual effects of the *Azolla* (Kolhe & Mittra, 1990, Ventura & Watanabe, 1993).

Dry matter production in a plant is a result of the length of production period and the

rate of dry matter accumulation in that period (Beadle, 1995). A lack of nutrients in the soil may result in early maturation of the plant, although in this study, it only resulted in less accumulation of dry matter by SC grown plants (Fig 2, Table 4). Studies on the yield of mungbean (Ram *et al*., 1994) and rice (Lumpkin & Plucknet, 1982, Joy & Havanagi, 1988, Watanabe & Liu, 1992, Kondo *et al*., 1989 Rengel *et al*., 1999) found yield increases with the addition of *Azolla* to the soil. In both the preliminary and final study, total above ground biomass yields were increased in all *Azolla* sand treatments (Figs. 1, 2 & 6). When the fertilisation rate was increased from 20% to 80%, the dry biomass of the wheat plants also increased. The preliminary study results showed that an increase (20% to 80%) in the amount of fresh and heated *Azolla* added per volume of sand had a significant effect, yet for the dry *Azolla*, the amount of fern added per volume of sand made no significant difference to the total above ground biomass (Fig. 2, Table 4). In the main experiment, significant differences in total above ground biomass were observed at the 50, 75 and 128 day harvests between the 20% and 80% *Azolla* sand treatments (Fig. 6, Table 19). This difference in the dry biomass between the preliminary and final study may have been due to the sample size differences. It may also be due to the differences in the time of harvesting of *Azolla*. When topsoil was used, the effect of the fertilisers in topsoil on the total above ground dry biomass was greater than that in the control (Fig. 7) but the difference was lower than that observed in sand. In sand (Fig. 6, Table 19), the straw biomass was greatly affected by fertilisation especially in the S80 treatment. In top soil (Fig. 7, Table 21), the fertilisers had no significant effects on the straw biomass.

The use of *Azolla* in lowland agriculture has been compared to use of legumes as well as inorganic fertilisers. Watanabe & Liu (1992) compared the incorporation of Urea (57 N ha^{-1}), *Azolla* (84 kg N ha^{-1}) and legumes (73kg ha^{-1}) and found rice yields to be 6 tons ha^{-1}, 6.6 tons $ha^{-1}$and 6.1 tons ha^{-1} respectively. The application of *Azolla* increases the N, P and K content of the soil and leads to improved crop yields (Singh & Singh, 1990; Kannaiyan, 1993). Lumpkin and Plucknett (1982) stated that 500 kg of fresh biomass gives 35-50 kg of dry biomass which contains N (1.2-2.4 kg), P (0.1-0.5 kg), K (0.6-1.3 kg) and organic matter (3.5-5 kg) which confirms the presence of N, P

and K in *Azolla*. In this study, no direct measurements were made on the presence of nutrients in the soil and crop yields but the response of the wheat growth parameters was used as a measure of the soil fertility. Ram *et al*., (1994) found that the addition of *Azolla* (24 tons ha^{-1}) to soil, increased ammonium and nitrate N resulting in optimum yields of pods, grain and plant weights of mungbeans (*Vigna radiata* L.). In pot grown *Nardus stricta*, the addition of N, P and K (3:1:2) significantly increased above ground biomass (Hartley & Amos, 1999). In the preliminary experiment, it was observed that the addition of fresh, dried and heated *A. filiculoides* to sand increased the grain weights, dry biomass, culm length and leaf areas of the wheat plants (Tables 17 & 27). In the main experiment, the addition of dried *A. filiculoides* increased wheat yields in sand (Fig. 6) but had less effect in topsoil (Fig. 7). Throughout the growth period, above ground biomass harvested from inorganically fertilised sand was as good as that in 20% *Azolla* treated sand (Fig. 6). This is similar to a study by Galal (1997) who used isotope labelling to estimate efficiency of *Azolla* and urea as N sources and agrees that *Azolla pinnata* is equivalent to the use of urea in a clay loam soil under rice cultivation. Furthermore, Talley and Rains (1980) incorporated 40 kg N ha^{-1} of dry *A. filiculoides* with spring rice in 1977 and obtained results equivalent to the use of ammonium sulphate, which supports the results in this study in which the biofertiliser increased dry biomass as much as the inorganic fertiliser or even better in nutrient poor soils (Fig. 6, Table19). In the main experiment, 80% *Azolla* in sand had the best effect and increased the above ground biomass by 220% and 342% at the 75 and 128 day harvests respectively in comparison to the control. Similar trends were observed in straw yields throughout the growth period (Fig. 6). The use of *Azolla* as a biofertiliser has been found to be comparable to legume use due to its high N content. The improved dry matter accumulation by wheat plants in comparison to the control in sand can be attributed to the increase in the amount of N available to the plants.

For notably significant effects, greater amounts of the biofertiliser need to be added and this may be over a long term depending on the soil (Mahapatra & Sharma, 1989, Watanabe & Liu, 1992). This may explain why the wheat plants in all topsoil treatments did not show significant differences in the dry biomass accumulation (Fig.

7, Table 21) at the 75 day harvest, as observed in the sand treatments (Fig. 6, Table 19). Significant differences in above ground and straw biomass in topsoil treatments were observed at the 50 day harvest whereby the plants grown in the control and 20% *Azolla* had similar above ground biomass to those plants harvested from all the sand fertilised treatments. This would suggest a higher nutrient availability in the inorganic and 80% *Azolla* topsoil treatments with higher effect on straw biomass. However by the 128 day harvests, the best average plant above ground biomass harvested was from S80 and T20 respectively.

In a perennial grass, *Molinia,* increase in N nutrition (0 to 20 g N m^{-2} yr^{-1}) resulted in an increase of above ground productivity (Aerts, 1994). In this study, this was notable in the sand treatments but not in the topsoil treatments (Figs. 2, 6 & 7). Although, S80 plants flowered later than S20 plants (Table 20), the S80 plants had the highest above ground biomass which is similar to the study on *Molinia* in which no detectable change in the rate of dry matter accumulation was found between the vegetative and reproductive stage. The increased above ground dry biomass was a result of increases in the straw and grain due to N application especially in the sand-fertilised treatments (Ewert & Honermeier, 1999). All plants in the topsoil treatments flowered at the same time (Table 20) and their final biomass does not differ as much as that of the sand treatments (Fig. 7). This shows that flowering was not affected by the addition of fertilisers to topsoil.

4.2 Grain yield

Although the N content of the harvested plants was not tested in this study, the improved response of above ground matter and grain is assumed to be partly caused by the presence of N as a nutrient that plants take up after the decomposition of the biofertiliser or as supplied by the inorganic fertiliser. The highest yield (grain and plant height) in field grown winter wheat was recorded with 210 kg N ha^{-1} and N application affected the accumulation of biomass up to heading (Delogu *et al*., 1998). In this study , the highest grain yield was obtained with the addition of 80% *Azolla* in sand and 20%

Azolla in topsoil (Fig.8, Table 28). However, during the preliminary study, the highest grain yields were obtained in the 80% heated *Azolla* in sand (Tables 6, 7 & 17). N uptake during grain filling was correlated to N applied to wheat (Delogu *et al*., 1998, Austin *et al*., 1977) which would suggest that in this study, the supply of N in fertilised topsoil was nearly as good as that in the topsoil control (Fig. 8H). This fact may be a result of sufficient nutrient status in the topsoil or poor nutrient release by dried *Azolla* in topsoil. N is a key element in achieving high yields in cereals since it is needed for the metabolic processes and its rate of uptake is determined by supply and demand (Delogu, *et al*., 1998). Delogu *et al*., (1998) state that soil N must be high at heading and grain filling for enhanced weight and high grain protein content. This may be the reason for a high average grain weight for the S80 grown plants (Fig 8D). These plants flowered later (Table 20) and therefore more nutrients may have been available in the sand for both heading and post heading. Plants grown in SH80, S80 and T20 appear to have had sufficient nutrient supply during plant growth up to heading, especially N which has a dominant role in dry matter accumulation and N (Austin *et al*., 1977 Delogu *et al*., 1998). Austin *et al*., (1977) found that N present in plants at anthesis and at maturity was strongly correlated with total above ground weight at these times. Therefore, the plants with the highest dry biomass would have the highest content of N which would be in the S80 and T20 grown plants in the main experiment (Table 28).

For a farmer, the grain is a very important part of the wheat plant, although the measurement of below ground matter is also useful. Spikelet number per ear and floret number per spikelet are important as it determines grain number and yield. Ewert and Honermeier (1999) investigated the duration of spikelet initiation and found that in winter wheat (cv. Taras), it was not affected by N application (200 kg N ha^{-1}) but the number of spikelets per ear was increased. This agrees with current studies whereby biofertilisation increased the grain yield per plant. Spikelet abortion was not prevented by N application (Ewert and Honermeier, 1999) a finding that may explain some results in this study on *Azolla* biofertiliser. However, in both the main and final experiments, the application of *Azolla*, led to an increase in the number of spikelets

per ear relative to the controls. The application of N generally results in more grain production until some other factor, such as moisture or phosphate, becomes limiting (Marschner, 1995). In this case, the limiting factor may be nutrients. Most of the N that the plant takes up before grain filling stage is used for the growth of non-reproductive structures, after which it contributes to grain production. In winter wheat the concentration of grain N and shoot N decreased as the grain yield increased (Thompson and Woodward, 1994). The application of N fertiliser to winter wheat in Montana significantly increased the grain protein percentage and bread loaf volumes (MacNeal *et al*., 1971). MacDonald (1992), found that grain weight and protein were affected by N application which is observable in the sand treatments but is not as clear in the topsoil treatments. Therefore, in SC and topsoil treatments, grain differences may be due to lower supply of assimilate from source tissues or a slower rate of utilisation of available assimilate within the grain.

The number of seeds or fruits and flower initiation can be affected by mineral nutrition which is clearly the case with various micronutrients (Rengel *et al*., 1999). In wheat, copper deficiency affects the reproductive phase as it inhibits anther formation, but hardly affects straw production. Therefore the poor grain formation in the SC plants, as compared to the fertilised plants (Fig 8D, Table 6), could have been a result of low micronutrient levels in the soil. Boron is essential for pollen tube formation and a low supply inhibits flowering and seed production (Marschner, 1995). A limited supply of mineral nutrients available for re-translocation from source to sink may affect grain yield rather than a limited carbohydrate source. The average grain size of a plant grown in sand treatments was not different from that of plants grown in topsoil. However, the number of grains per plant and therefore the total grain weight per plant differed between treatments (Fig 8, Table 7). The increase in biofertiliser from 20% to 80% *Azolla* resulted in significant grain weight increases per plant. The SNPK grown plants had more grain than the S20 plants but less than the S80 plants which makes the S80 treatment better in sand than the inorganic fertiliser (Fig. 8D). Topsoil grown plants had similar values of grain weight per plant and may therefore appear to have had sufficient micronutrient supply (Fig. 8H).

4.3 Relative growth rate (RGR)

As the plant grows the non-growing and non-photosynthetic material increases leading to a decline in relative growth rate (Street and Opik, 1970) which was observed in all treatments in the preliminary and final experiment (Fig. 9 & 10). During the first 50 days, there is a high relative growth rate due to the addition of mass in form of growing points and photosynthetic area. Most of the dry matter was accumulated during this period. The period of dry matter accumulation was the same in all treatments without any differences in RGR between treatments over a particular time interval. Gomez-Macpherson *et al*., (1998a) found no difference in RGR despite large differences in development rate in wheat lines within each isogenic set and photoperiod. Furthermore, the lack of difference in RGR calculated using above ground biomass was attributed to an increase in net assimilation rate in the early lines. No difference was found in isolines RGR despite phenological differences in spaced plants. The lack of significant difference in relative growth despite differences in flowering and leaf areas may be due to a higher photosynthetic efficiency in the later flowering plants in the above ground biomass (Gomez-Macpherson *et al*., 1998b). However, in this study, the greater leaf area did not result in greater relative growth rate despite the increases in biomass due to fertilisation.

4.4 Leaf Area (LA)

In a study on winter wheat, the dry weights of leaves and stems at anthesis and maturity was increased by N, as was leaf area (Pearman *et al*., 1977). In winter wheat, Austin *et al*. (1977) found a strong correlation between dry matter accumulation and N content which are affected by photosynthetic carbon fixation. N affects the dry matter since it has an effect on leaf area and therefore the response of plant leaf areas is important. In the preliminary experiment, at 20% and 80%, the heated treatment effected a significantly higher leaf area on wheat plants (Fig.3, Table 11) and more above ground biomass accumulation. In the main experiment, all fertilised sand treatments effected significantly greater leaf areas on the wheat plants than the SC

treatment (Fig. 11) with significantly greater above ground biomass accumulation. In topsoil (Fig. 12), the T80 and TNPK effected significantly greater leaf areas on wheat plants but did not result in the most above ground biomass accumulation among the topsoil treatments.

Vouillot and Devienne-Barret (1999) found that N accumulation and translocation between the organs of vegetative winter wheat was affected by soil N availability. Furthermore under N deficiency, young leaves grew by utilising N remobilised from older leaves but under fertilised N conditions both soil and remobilised N contributed to leaf growth. This may explain the differences in leaf areas between the control plants and the fertilised plants in this study. This would mean that SH80 and S80 which had the highest leaf area, had a high supply of soil N and remobilised N. Among the factors required for plant growth are mineral nutrients and an increase of these (from the deficiency range), will increase the growth rate to an optimum value. Supply at super optimal levels reverses increased productivity and mineral availability interacts with water availability (Marschner, 1995). However throughout this study, water was not a constraint, and increased leaf areas that contributed to increased above ground biomass can be attributed to the availability of mineral nutrients.

Langer (1966) agrees that the addition of nutrients to cereal crops affects leaf area and numbers. Investigations on a perennial grass (*Molinia)* showed that N allocation to leaves increased with N supply (Aerts, 1994). In wheat, Wojcieska (1994) found that the growth was strongly affected by the N nutrition in terms of dry weight and dry matter distribution with beneficial effect on the size of the leaves, a phenomenon that was observed in both the preliminary and final experiments on the biofertilisation with *Azolla*. The 20% *Azolla* treatments had similar effects on leaf areas of the sand and topsoil grown plants (Figs. 11 &12, Table 23). This is in accord with a study by Baxter *et al*. (1994), on montane grasses. The plants harvested from SH80 and S80 had the largest leaf area and accumulated the greatest dry biomass. T80 grown plants had the greatest leaf areas among the topsoil treatments but T20 grown plants accumulated the most biomass with less green maximum leaf area. In both topsoil and sand

treatments, plants fertilised with 80% and inorganic fertiliser had the greatest leaf areas (Table 23).

It is important to note that Gomez-Macpherson *et al*. (1998a) found that mature leaf blade size was associated with delayed floral initiation. Aerts (1994) found that on the whole, late developing wheat had the largest total leaf area per plant. Among the sand treatments in this study, the S20 grown plants flowered earliest and attained their maximum green leaf area earlier than the S80 and SNPK grown plants. However, fertilisation did not appear to affect the time of flowering and leaf areas in topsoil treatments.

4.5 Specific Leaf Area (SLA)

Specific leaf area as a measure of the accumulation of carbon per unit of green leaf area will decrease with time as the leaves mature (Beadle, 1993), with lower values for the more efficient carbon-investing plants. Plants with higher SLA should have a higher RGR (Beadle, 1993). In wheat, Gomez-Macpherson (1998a) found that a decline in RGR was associated with a decline in LAR. This is similar to results from the preliminary study in which RGR and LAR (Fig. 4) declined over time while in the main study RGR (Fig. 10) and SLA (Fig. 13) showed the same trends in all treatments.

Aerts (1994) found that *Molinia*, a highly productive grass species, allocates most of its biomass to the roots but compensates the low biomass allocation to the leaves by a high SLA. In a study on herbaceous plants, Meziane and Shipley (1999), found that SLA was affected by both irradiance and nutrient supply. This differs from the main experiment in which there were no observable significant differences in SLA in all treatments. Therefore the increases in leaf areas did not result in more efficient dry matter accumulation in either sand or topsoil, although differences were observed at the 50 and 75 day harvests.

4.6 Root biomass

Prasad and Ram (1982) recorded increased dry root biomass of wheat plants at 62 days due to the application of *Azolla* to alluvial soil. Root biomass was found to increase with increase in *Azolla* application to a value of 100 tons ha^{-1}. In pot experiments, it was found that the addition of N, P and K fertiliser increased the root biomass of *Nardus stricta* (Hartley & Amos, 1999). Findings by Aerts (1994) show that biomass allocation to roots in *Molinia*, a heathland grass species, was lower at high than at low N supply. In this study, increased root biomass in S20, S80 and SNPK could be the result of increased N supply in *Azolla* and inorganic fertiliser treatments (Table 26).

Split pot experiments showed that at 80% *Azolla* biofertilisation rate (Table 16, 26), there is a saturation of nutrients that contributes to increased root branching and thus improves plant nutrient uptake. Sand was used because it is easier to harvest the roots although the presence of the *Azolla* made it difficult to remove all the roots and so the results may not be accurate due to loss of root material in the sand-*Azolla* treatments. In the split pot experiments, significantly more roots were collected from the *Azolla* side than from the pure acid washed sand. The root biomass collected from the sand treatments in the main experiment showed more root biomass in the inorganic and *Azolla* fertiliser treatments than in the control (Tables 25 & 26). In the preliminary experiment, R:S ratios of SC plants were higher than the R:S ratios of plants harvested from dried *Azolla* treatments (Table 14 & 15). In the main experiment, R:S ratios were highest in the SC treatment (Table 26) suggesting a higher investment in their underground biomass than in the above ground biomass.

4.7 Soil moisture

Due to the high amount of organic matter added to a soil by the incorporation of *Azolla*, soil fertility was improved (Van Hove, 1989, Singh & Singh, 1990, Lumpkin & Plucknett, 1982, Ventura & Watanabe, 1993). Ram *et al*. (1994) found that the addition of *Azolla* to a sandy loam soil (pH 7.8) improved soil physical properties and

water holding capacity. In a long term rice-wheat cropping, green manure *Azolla* proved to be as good as inorganic fertiliser and improved soil structure (Mubarik, 1999, Ventura and Watanabe, 1993). This was observed in the sand treatments in this study (Figs. 5 &14). Mubarik (1999) found that *Azolla* increased the water holding capacity of soil. In both the preliminary and final study, the incorporation of *A. filiculoides* in sand supplied nutrients and increased the water holding capacity of sand. The best soil-water content was observed in the preliminary study in the SH80, SF50 and SF80 *Azolla* treatments (Fig. 5). This would be an important factor under field conditions for nutrient poor sandy soils. Topsoil treatments had different results from those observed in the sand treatments. There were no significant effects on soil water content between the control and fertilised the topsoil treatments. The soil water content in the 20% and 80% *Azolla* sand treatments was as good as that in the topsoil treatments (Fig. 14).

Previous studies and this study show that *A. filiculoides* increases above ground biomass, root ramification, grain weight, leaf area in crops. As an organic fertiliser, it also improves soil properties. Although it has mainly been applied as a green manure, Ram and Prasad (1982,1983), and results from this study do confirm that *Azolla* can be used under dryland conditions to compliment and substitute inorganic fertilisers.

4.8 Potential problems

Sporulation

Spore formation by *Azolla* has been found to be associated with mat senescence (Ashton, 1982, Watanabe & Ramirez, 1990). Ashton (1982) determined that *A. filiculoides* spores take a period of 17 to 43 days to germinate in the laboratory after the artificial combination of megaspores and microspores. Janes (1998b) found that in Britain, at 20°C, *A. filiculoides* spore germination took 11-25 days while at 15°C, it took 40-63 days. During the main experiment, unlike in the preliminary one, the spores germinated following extraction from both sand and soil treatments. The spores in

sand took 74 days to germinate, while those in topsoil took 96 days to germinate. In the preliminary study, spore germination may not have occured due to absence of spores in the *Azolla* used as a result of the time of harvest. Harvesting might be ideal during spring or early summer before the multi-layered mats are formed by the *Azolla* plants (Ashton, 1982). When harvesting, care must be taken to choose mats that are green since the red mats may contain spores and lower concentrations of N and P concentrations (Janes, 1998a, Watanabe & Ramirez, 1990).

In the preliminary study, the length of time may not have been enough for spores to germinate. This would suggest that the spread of *Azolla* when used in biofertilisation is very unlikely. In view of the germination of spores during the main experiment, a problem arises in the use of *A. filiculoides* as a biofertiliser.

Heavy metal release into soil

Several studies have showed that *Azolla* takes up heavy metals especially from polluted waters (de Wet *et al*., 1990). *Azolla* as fresh and dry matter is known to take up heavy metals such as Zn, Cu, Cr, U and Hg. (de Wet *et al*., 1990, Sela *et al*., 1988, Jain *et al*., 1989, Zhao & Duncan, 1997, Mishra *et al*., 1987, Rengel *et al*., 1999). This would affect the use of *Azolla* as a biofertiliser since plants would take up the metals if made available in the soil water. It would necessitate the testing for metals in the *Azolla* before application into soil. However the uptake of metals like mercury is dependant on their concentration in water (Mishra *et al*., 1987). The uptake of metals was found to affect the health of the fronds making their lifespan very short and causing them to change colour from green to red (Sela *et al*., 1988). Furthermore, the presence of metals in *Azolla* decreased the nutrient (Mg & K) value of the plants (Sela *et al*., 1988). *Azolla* was found to be more resistant to iron and copper uptake than *Lemna minor* L. (Jain *et al*., 1989). Therefore release of metals into soil by *Azolla* and uptake of metals by crops needs to be investigated before large scale use of *Azolla* as a biofertiliser when metal pollutants are likely to be present. The source of the *Azolla* is thus important and must not be from water bodies in industrial areas.

4.9 Conclusion

In a review on *Azolla*, Wagner (1997) calls *Azolla* “a green gold mine.” This fact cannot be ignored with regard to substituting it for inorganic fertilisers in order to conserve the environment as well as maintain sustainable agriculture. The findings from previous studies agree with results from both the preliminay and main studies presented in this thesis. This would justify the use of *Azolla* as a biofertiliser in nutrient poor soils. Addition of *Azolla* does increase wheat yields. Although, it was only found to be significant in sand, the use of *Azolla* resulted in notable increase in yields in topsoil. Therefore, the use of *Azolla* as a biofertiliser in the absence of anaerobic conditions holds potential as a supply of nutrients for crops. For nutrient sufficient soils, long-term trials need to be carried out, as well as perhaps increasing the amounts of *Azolla* applied.

However more studies, especially field trials, need to be done on the decomposition of *Azolla* under dryland conditions in a range of soils. The *Azolla* could be applied two weeks before planting and again later during the growth period of the crop. Another option would be the use of *Azolla* as the first fertiliser application and then followed later by the application of inorganic fertiliser so that the use of inorganic fertiliser is halved. This has the potential to reduce the use of inorganic fertilisers in a farming system.

The problems of sporulation and heavy metal accumulation and subsequent release by *Azolla*, need to be addressed before field applications by the farmer can be made confidently. Spores in this study were found to germinate in mud that had been removed from both sand and topsoil growth media. This poses a problem to water bodies since *Azolla* is a weed. The problem of viable spores could be partly solved by harvesting at the right time considering the fact that *A. filiculoides* was found to undergo sporulation starting in September to February after mature mat establishment (Ashton, 1982). In places where *Azolla* does not occur, introduction to pristine water

bodies would be needed for easy access for harvesting. This practice is likely to be controversial, and a person undertaking such measures would have to take strict precautions to ensure that the weed does not spread within and between water systems.

Future research could focus on testing the harvested biomass (straw and root) and concentration of nutrients and heavy metals per unit grain weight. This is essential due to the fact that metal uptake by the crops poses a danger to human and animal consumption (Rengel *et al*., 1999).

From the preliminary and final studies, the application of heated or dried *Azolla* improves wheat yields on nutrient poor sandy soils and holds potential for use as a biofertiliser if the above problems are investigated and solutions found. To improve yields in a sandy loam topsoil of an average pH 4.1, an amount of 8.14×10^3 kg *Azolla* ha^{-1} is required. Although costing was not done for either the preliminary or final study, in a field study in Madagascar, the annual budget of a project using *A. pinnata* as a biofertiliser was US$80,000 which was equivalent to the importation of 80 tons N and would save immensely on foreign exchange (Van Hove *et al*., 1994). One must bear in mind that a well maintained *Azolla* mat produces 30 kg N ha^{-1} (Lumpkin and Plucknett, 1982) The use of *Azolla* as a biofertiliser could save on input of inorganic fertilisers as well as being being environmentally friendly with short and possibly long-term effects on crop yields.

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