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The Response of Nitrogen Biofertilizer *Azolla* to Herbicides

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Abstract

The water fern *Azolla* used as rice nitrogen biofertilizer was tested to characterize its sensitivity, to common herbicides used for rice farming in Central America. Experiments with *Azolla filiculoides* and *A. caroliniana* were conducted under controlled growth conditions of temperature and day-night regime and in outdoor ponds. *Azolla* was grown in nitrogen free medium and tested after one week for the effects of the herbicides. We analyzed plant growth, N_2-fixation, tested by acetylene reduction assay (ARA), protein, chlorophyll and sugar content. The hormonal herbicide 2-methyl-4-chloro-phenoxyacetic acid (MCPA) was found compatible at the concentration of 1–3 mg/l. Pendimethalin (inhibitor of cell division) and fenoxaprop-p-ethyl (inhibitor of fatty acid biosynthesis) were compatible for both *A. filiculoides* and *A. caroliniana* at the concentration of 3 mg/l in experiments conducted indoors and outdoors. Fenoxaprop-p-ethyl was most favorable for N_2-fixation. These results indicate that the standing crop and biomass of *Azolla* as a potential nitrogen biofertilizer is compatible with pendimethalin and fenoxaprop, as practical weed control agents for rice farming.

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1. Introduction

The aquatic fern *Azolla* and its nitrogen fixing cyanobiont *Anabaena azollae* are used as a nitrogen biofertilizer for rice farming world wide, mainly in the Far East (Talley et al., 1977). Farmers in hot and humid tropical countries, use herbicides in large quantities for weed control in rice fields, thus, *Azolla* as rice associated susceptible plant, is exposed to harmful herbicides.

Only few studies were reported on the influence of herbicides on *Azolla* growth and nitrogen fixation. Singh et al. (1988) tested the effect of some herbicides such as 2,4-D and pendimethalin, on *Azolla*. They concluded that pendimethalin and 2,4-D can be applied to *Azolla*. Toia et al. (1981) tested the effect of propanil and 2,4-D on *Azolla*, and found that propanil at the concentration of 1 mg/l and 2,4-D at concentration of 100 mg/l hindered completely the growth of *Azolla*. Srinivasan et al. (1993) studied the effect of some rice herbicides on *Azolla pinnata*. They concluded that by waiting until three days after application before inoculating the fields with *A. pinnata*, the effects of herbicides decreased.

Application of *Azolla* as biofertilizer, shading the water surface of flooded rice brings to reduced amounts of herbicides needed, by impeding growth of some weeds (Nierzwicki-Bauer, 1990).

The aim of this research was to examine the effect of some herbicides on *Azolla* growth, its physiology and to select compatible herbicides for *Azolla*, not harmful for nitrogen fixation and its cyanobiont *Anabaena*. Experiments with *A. filiculoides* and *A. caroliniana* were conducted under controlled growth conditions of temperature and day/night regimen and in outdoor ponds. Our results of the standing crop and nitrogen fixation of *Azolla* in a model system and outdoor experiments treated with pendimethalin and fenoxaprop-p-ethyl suggest that *Azolla* can indeed function as effective biofertilizer in rice fields treated with these compatible herbicides.

2. Materials and Methods

Azolla filiculoides and *A. caroliniana* were grown in the phytotron under controlled temperature, day: night of 27:22°C and 16:8 hr day:night regime. The *Azolla* was grown in plastic containers in nitrogen deficient solution, International Rice Research Institution (IRRI) (Watanabe et al., 1977), supplemented with different concentrations (mostly higher to those used in rice fields) of the tested herbicides. *Azolla* was harvested after one or two weeks of growth. The outdoor experiments (2–3 weeks) were conducted in 1 m × 4 m plastic ponds filled with 300 m^3 IRRI growth solution. The ponds were covered with polyethylene for the plants protection against the winter cold.

Herbicides used: imazapyr, pendimethalin, fenoxaprop-p-ethyl, MCPA, 2,4-D, and propanil. All herbicides were obtained from stocks of our herbicide collection (Plant Science Institute).

Enzyme analysis

Nitrogen fixation (nitrogenase activity) was measured by acetylene reduction to ethylene (ARA) (Nierzwicki-Bauer, 1990). *Azolla* plants in 2 ml IRRI growth solution were introduced into rubber stoppered 40 ml serum bottles. Acetylene (10 percent of the gas phase) was injected through the rubber stopper, and the samples were incubated for one hour at 28°C under light intensity of 100 µEinstein/m^2 × s. A 4 ml sample from the gas phase was injected to a gas chromatograph (Vega 6000 GC, Carlo Erba Instruments). Nitrogenase activity was calculated as µl $C_2H_2 \times h^{-1} \times$ g fresh weight (FW) of *Azolla*.

Glutamine synthetase (GS) activity in *Azolla* extracts was measured by the synthetase assay according to Shapiro and Stadman (1970). *Azolla* extraction was conducted in 50 mM buffer phosphate pH-7.3 with 10 mM DTT (dithiothreitol) and 1% PVP (polyvinylpyrrolidone) according to Ray et al. (1978). Results of glutamic acid gamma-mono-hydroxamate production were expressed as nkat GS/gFW.

Chemical and biochemical analyses

Chlorophyll content of *Azolla* was measured after extraction in 80% acetone. Chlorophyll absorption was measured at 663 nm and 645 nm, according to (Arnon, 1949). Soluble protein content was measured according to Bradford (1976). Total sugar was measured according to Dubois et al. (1956). Reducing sugars were measured according to Samner's method (Bernfeld, 1955). Ammonia in growth medium was determined with Nessler reagent according to Hill-Cottingham and Wagner (1962).

Statistics

Experiments were repeated at least three times giving similar results. Data represent means and standard deviations of three replicates per treatment of a representative experiment.

3. Results and Discussion

A number of herbicides, effective in different mode of action, were introduced, to follow their influence on *Azolla* productivity, nitrogen fixation, glutamine synthetase, protein, chlorophyll and sugar content. The experiments were conducted under controlled growth conditions in the phytotron, in order to obtain quantitative dimension of the effect of each herbicide.

Effect of pendimethalin

The adsorption of pendimethalin to the plant roots as described by Hess (1989) and Devine et al. (1993), were observed in our experiments with *Azolla* plants. This herbicide is known to inhibit cell division, by harming the microtubuli, and thus is not harmful to prokaryotes. Our assumption was that *Anabaena*, the cyanobiont of *Azolla* and its nitrogen fixation activity will not be inhibited by pendimethalin. In the presence of low concentration of pendimethalin up to 5 mg/l, there was little effect on *A. filiculoides* growth (not shown), pendimethalin at 10 mg/l and higher concentration, was effective, roots were slightly blackened, deformed and swollen at the root tips and growth was inhibited within treatment for one week (Table 1).

Table 1. Effect of pendimethalin on indoor (phytotron) growth of *A. filiculoides*, ARA, and sugar content. In this and the following tables data represent three replicates and standard deviations are given.

Pendimethalin (mg/l)	Growth-fresh weight (% of control)	ARA (µl C_2H_4/g FW/hr)	Total sugar content (mg/g FW)
0	47 ± 1.7	3.5 ± 1.06	28.0 ± 3.25
10	19 ± 2.4	5.0 ± 1.89	32.0 ± 5.6
30	12 ± 2.4	30.0 ± 1.84	37.5 ± 4.1

Table 2. Effect of pendimethalin and MCPA on ARA and reducing sugar content in *A. filiculoides*.

Treatment	ARA (µl C_2H_4/g FW/hr)	Reducing sugar (mg/g FW)
Control	13 ± 4.1	0.30 ± 0.14
Pendimethalin (5 mg/l)	30 ± 4.1	0.69 ± 0.14
MCPA (2 mg/l)	24 ± 7.35	0.70 ± 0.01

Nitrogenase activity tested by ARA, was higher in treated plants (Table 1). Total sugar contents were higher in the treated plants (Table 1), while chlorophyll and protein contents were not affected significantly (not shown).

Aiming to resolve the relation between high levels of sugars and high ARA activity, an experiment using 5 mg/l pendimethalin was performed. After a week of treatment reducing sugar content and ARA activity were 50 percent higher in treated plants (Table 2).

A similar effect was obtained with 2 mg/l MCPA (Table 2). A comparative analysis of shoots and roots of *Azolla,* treated with 5 mg/l pendimethalin for a week, showed significantly higher levels of sugars in both the shoots and the roots of treated *Azolla* plants (Fig. 1). These results indicate that photosynthesis and carbon metabolism was not inhibited by pendimethalin.

Furthermore, inhibition of cell division by pendimethalin, allowed the accumulation of soluble sugar in the shoots and roots. Thus, the accumulation of sugar in the *Azolla* shoot contributes to enhanced nitrogenase activity.

We have tested the recovery of *A. filiculoides* after exposure to high pendimethalin concentration of 10 mg/l for one week. The treated plants were then transferred to fresh IRRI solution, with no herbicides, for an additional week. Growth of *Azolla* fronds was observed at the apex, and newly developed healthy roots were visually observed in plants treated with 10 mg/l.

These results show that pendimethalin can be used as a pretreatment prior to rice planting as suggested by Singh et al. (1988). Even if limited damage will occur, due to the treatment of herbicide to *Azolla,* after a week or two, there will be a recovery of the *Azolla* crop and it will take place once the herbicide is decomposed (UV degraded) or diluted out with the constant refilling of the ponds. Structural studies with a scanning electron microscope showed no damage compared to the control (not shown), to the *Anabaena* in *A. filiculoides,* grown in 10 mg/l (not shown) and 50 mg/l pendimethalin (Fig 2). ARA and total sugar results at concentration of 50 mg/l pendimethalin, were similar to values at 10 and 30 mg/l (not shown), indicating that the cyanobiont under these high herbicide concentrations was not affected.

Heterocyst frequency of the cyanobiont *Anabaena azollae,* was tested in *A. filiculoides* treated with 5 mg/l pendimethalin and 2 mg/l MCPA. No differences in heterocysts frequency were detected between herbicide treated *A. filiculoides* and control plants, indicating that heterocyst differentiation was not the cause for higher ARA activity.

Effect of fenoxaprop-p-ethyl

Fenoxaprop-p-ethyl is known to inhibit fatty acid biosynthesis and phospholipid membrane synthesis (Devine et al., 1993). High concentrations of fenoxaprop-p-ethyl (10–30 mg/l) caused fast mortality of *A. filiculoides* within few days (not shown), therefore we conducted experiments at concentrations of 1–3 mg/l. After a week treatment with 1 mg/l and 3 mg/l fenoxaprop-p-ethyl, growth of *Azolla* was decreased by 50 percent, while nitrogen fixation (ARA) was not hindered. After two weeks, of *Azolla* treatment with 1 and 3 mg/l, ARA was decreased by 33 percent and 80 percent respectively (not shown). Chlorophyll content was not affected by the herbicide (not shown) and protein content was significantly higher in treated plants (Table 3).

The effect of pendimethalin and fenoxaprop-p-ethyl on *A. caroliniana* was similar to the results with *A. filiculoides*. After one week of growth in IRRI solution, and in the presence of 3 and 10 mg/l of pendimethalin and fenoxaprop-p-ethyl, inhibition to *Azolla* growth was observed in the presence of 10 mg/l of both herbicides. Nitrogenase activity was inhibited at 10 mg/l of both herbicides. Sugar content increased substantially with pendimethalin at 3 and 10 mg/l concentrations, while fenoxaprop-p-ethyl did not affect sugar content. Protein content was not affected in *A. caroliniana* (not shown).

Table 3. The effect of fenoxaprop-p-ethyl on protein content in *A. filiculoides*.

Fenoxaprop-p-ethyl (mg/l)	Protein content (mg/g FW)
0	1.5 ± 0.08
1	1.8 ± 0.08
3	2.8 ± 0.17

Table 4. Effect of pendimethalin and fenoxaprop-p-ethyl on in vitro GS enzyme activity in *A. filiculoides* and *A. caroliniana*.

Azolla	Herbicide	GS activity (nkat/g FW)
A. filiculoides	None	1.22 ± 0.03
A. caroliniana	None	0.55 ± 0.08
A. filiculoides	Pendimethalin (3 mg/l)	1.36 ± 0.01
A. caroliniana	Pendimethalin (3 mg/l)	0.59 ± 0.01
A. filiculoides	Fenoxaprop-p-ethyl (3 mg/l)	1.16 ± 0.04
A. caroliniana	Fenoxaprop-p-ethyl (3 mg/l)	1.45 ± 0.14

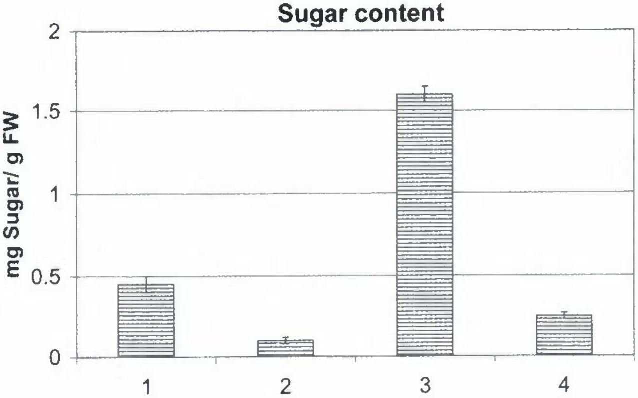

Figure 1. Effect of pendimethalin on sugar content in leaves and roots of *Azolla*. 1-control leaves, 2-control roots, 3-leaves with 5 mg/l pendimethalin, 4-roots with 5 mg/ml pendimethalin. Bars represent standard deviation of three replicates.

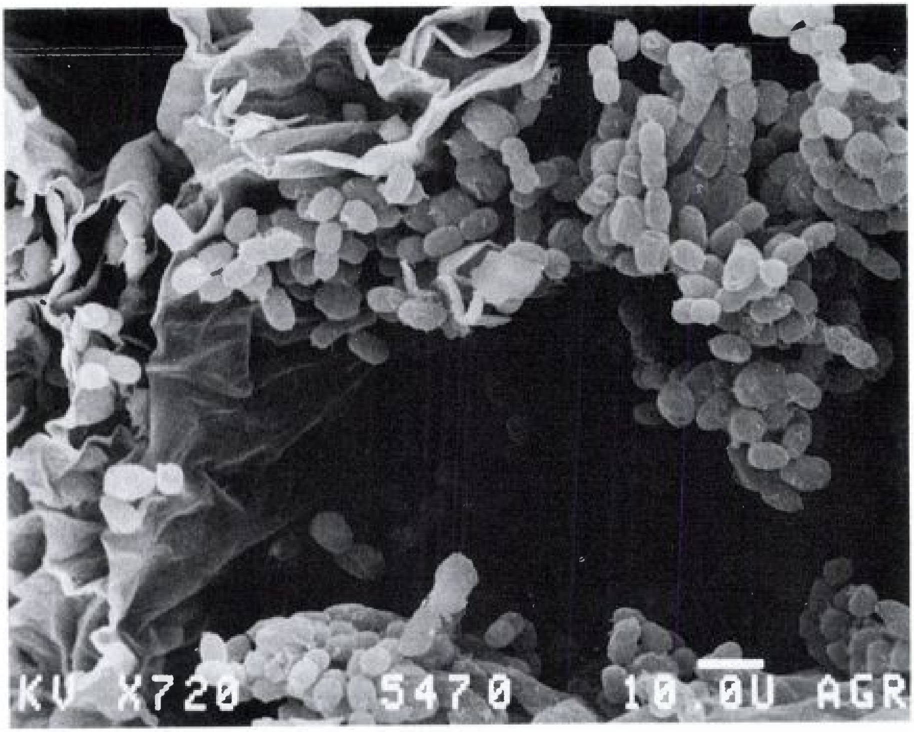

Figure 2. Electron micrograph of a mature leaf cavity of *Azolla* exposed to 50 mg/l pendimethalin for one week, demonstrating *Anabaena* cells and host hair cells.

Glutamine synthetase (GS) activity

Glutamine synthetase is a key enzyme in the assimilation of ammonia, and therefore was chosen to study pendimethalin and fenoxaprop-p-ethyl effects on its activity in *A. filiculoides* and *A. caroliniana*. Pendimethalin had no effect on GS activity in both *Azolla* species. Fenoxaprop-p-ethyl increased GS activity by three fold in A. *caroliniana*. GS activity in *A. caroliniana* was 50 percent lower than its activity in *A. filiculoides* (Table 4). No free ammonia was detected in the growth medium as a result of GS inhibition as measured with Nessler's reagent according to Hill-Cottingham and Wagner (1962).

Adaptation of Azolla to pendimethalin and fenoxaprop-p-ethyl

Herbicides are frequently applied in the field, therefore experiments of adaptation were conducted to find if *A. filiculoides* could be adapted to the herbicides and may acquire herbicide tolerance. For this purpose low concentrations of herbicides were applied as pre-adaptation treatment followed by higher concentration.

No adaptation was observed when pretreatments were conducted with 0.1 and 0.3 mg/ml fenoxaprop-p-ethyl and 1 and 5 mg/l pendimethalin, for two days failed to lead to herbicide tolerance. It is possible that longer adaptation periods could lead to adaptation to the herbicide.

Imazapyr

Imazapyr is known to inhibit the enzyme acetolactate synthase (ALS) involved in the synthesis of the branched amino acids valine, leucine and isoleucine (Ray et al., 1989). Growth of *A. filiculoides* was found to be very susceptible to low concentrations of up to 1 mg/l imazapyr. At 0.5 mg/l, ARA showed an increase of 174 percent over the control. This result was in accordance with sugar content which was doubled in the treated plant compared to control. These results are similar to those obtained with pendimethalin and may indicate the relation between high sugar content and the high energy demands for nitrogen fixation activity.

Under higher concentrations of 10–20 mg/l, we observed severe damage to *Azolla* growth and nitrogenase activity. Chlorophyll contents were lower than in the control, even at 0.5 mg/l. Protein content was slightly decreased with concentration above 5 mg/ml (not shown).

Propanil

Propanil, known to inhibit photosynthesis in the photosystem II reaction,

Table 5. Effect of pendimethalin and fenoxaprop-p-ethyl (3 mg/l), on ARA, reducing sugar and protein content of *A. filiculoides* grown outdoors.

Herbicide	Day of experiment	ARA ($\mu l\ C_2H_4$/g FW)	Reducing sugar (mg/g FW)	Protein (mg/g FW)
Pendimethalin				
0 mg/l	5	30 ± 4.9	0.31 ± 0.07	1.4 ± 0.08
	11	34 ± 10.2	1.10 ± 0.08	2.9 ± 0.41
	18	22 ± 8.1	1.70 ± 0.65	2.6 ± 0.32
3 mg/l	5	10 ± 5.2	0.26 ± 0.04	1.0 ± 0.09
	11	22 ± 4.1	1.50 ± 0.13	2.9 ± 0.22
	18	15 ± 4.1	1.60 ± 0.32	2.4 ± 0.17
Fenoxaprop-p-ethyl				
0 mg/l	7	45 ± 8.1	n.d.	4.6 ± 0.28
	14	40 ± 8.1	3.30 ± 0.04	4.1 ± 0.82
	21	18 ± 3.2	2.50 ± 0.41	3.7 ± 0.40
3 mg/l	7	50 ± 4.9	n.d.	4.9 ± 0.41
	14	45 ± 7.3	3.60 ± 0.49	4.9 ± 0.01
	21	24 ± 8.1	3.50 ± 0.42	3.9 ± 0.41

inhibited growth of *A. filiculoides* up to 50 percent at concentrations of 0.5–1 mg/l. At 1 mg/l propanil, ARA was inhibited up to 60 percent and sugar content was reduced by 50 percent. There was no effect of propanil on protein content.

2,4-D and MCPA

Both herbicides act like auxin hormones. The herbicides were tested in concentrations of 1–10 mg/l. There was a mild effect on *A. filiculoides* growth. One mg/ml of 2,4-D did not inhibit growth whereas 10 mg/ml caused a 20 percent reduction in growth. MCPA inhibited growth partially. Chlorophyll content was not affected by MCPA, but was slightly lower in the presence of 2,4-D, which also lead to the development of chlorotic plants. The growth of root hairs was enhanced at concentrations up to 3 mg/l. ARA was enhanced by the two herbicides at 1 mg/ml, and even doubled with 2,4-D. Protein content was slightly lower with 2,4-D and was not affected with MCPA (not shown).

The compounds 2,4-D and MCPA at low concentrations inhibited only slightly *Azolla* growth, did not inhibit ARA, and sometimes even enhanced it. These results lead to suggest the use of these two herbicides at low concentration with dense coverage of *Azolla* in rice field. However, these concentrations may not be effective to control the weeds in the rice field.

Effect of herbicides on outdoors grown A. filiculoides

Pendimethalin: At 3 mg/l, significantly decreased ARA to 33 percent after 5 days, but showed 50% recovery after 11 days. Sugar and protein content were not hindered (Table 5).

Fenoxaprop-p-ethyl: No damage to *Azolla* was observed and *A. filiculoides* biomass tripled within two weeks. ARA and protein content were slightly increased. Sugar content was higher in the treated plants (Table 5). These results suggest that fenoxaprop-p-ethyl at a concentration of 3 mg/l, could be used as a suitable herbicide with *A. filiculoides* (Table 5).

These results strongly suggest that both herbicides pendimethalin and fenoxaprop-p-ethyl at concentrations comparable to those used in the field are suitable for weed application in rice biofertilized with *Azolla*.

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