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Department of Agronomy and Soils, Pullman, Washington 99164-6420 / 509-335-3475

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Enclosed are two copies of the semi-annual progress report for project no. 6446, "Fingerprinting of Azolla Germplasm, grant no. DPE-5542-G-SS-6041-00.

Sincerely,

William J. Zimmerman

1

6446

FINGERPRINTING OF AZOLLA GERMPLASM (No. 6446)

January, 1988

W.J. Zimmerman[1], T.A. Lumpkin[1], and I. Watanabe[2]

[1]Department of Agronomy and Soils, Washington State University, Pullman, WA, USA; [2]Soil Microbiology Department, International Rice Research Institute, Los Banos, Laguna, PHILIPPINES

Summary

Final questions on the most efficient methodology for the enzyme electrophoresis of *Azolla* were answered. The potential for hybrid identification was proved. All three successful hybrids currently in existence were confirmed as hybrids. The biochemical taxonomy of the species in section Azolla is almost complete. Definite identification at the specific level is now possible using simplified techniques to facilitated use in LDC institutions. The absolute identification of individual accessions is possible in some but not all instances through enzyme electrphoresis.

Fingerprinting of Azolla Germplasm

Part 1

The results of our work on optimizing methodology for the enzyme electrophoresis of Azolla accessions had been mostly complete and given in our last progress report. They have now been submitted for publication. The key variables should be re-iterated briefly. They are type of tissue, stress, and contribution by the cyanobiont Anabaena azollae to zymograms.

These variables represent: 1) the liklelihood that new isolates collected in the field will be physiologically stressed, and 2) potential scientific objections to our methodology that needed to be addressed. Up to 11 enzymes were utilized for the study of each variable. The enzymes of leaf tissue were preferred, except where noted, using our previously reported techniques.

The most common environmental stresses, that of phosphorous deficiency and nonfavorable temperatures, were chosen to test their effects on the isoenzyme results of selected Azolla species. These suboptimal conditions resulted in diminuitive fragile fronds. The major discernable difference in enzyme banding patterns was a greatly reduced staining intensity. Stressed accessions did not yield results for several enzymes, but missing bands were only rarely apparent. Our recommendation is that unknown strains collected in situ should be stabilized in

a moderate controlled environment prior to isoenzyme analyses.

Root tissue was analysed as a symbiont-free alternative to leaf tissue. This choice of tissue is more tedious to harvest and grind, but would circumvent any problem of cyanobiont contribution to a zymogram. Successfully discerned enzymes were not always identical to leaf tissue, and yielded poorer resolution with our methodology. No attempt was made to expand our research soley to optimize resolution of the root enzymes. We do not recommend their use for enzyme comparisons among accessions, only for comparison, if desired, of leaf vs. root differences.

The contribution of the cyanobiont to zymograms was also examined by analysing Azolla filiculoides strains with and without Anabaena. These strains came from the IRRI collection. Only slight incongruities appeared in banding patterns, possibly due to some physiological change in the Azolla upon loss of its cyanobiont. These only involved minor bands. The conclusion was that the contribution of Anabaena to zymograms of leaf tissue was insignificant, barring intense overstaining of enzymes. Thus any variability in the cyanobacterium will not result in adding uncertainty to interpretation of the banding patterns of the host enzymes by our techniques.

Part 2

Based upon our previous enzyme overview among the species of Azolla, we had observed the lower percentage of similarity in section Azolla (formerly Euazolla) than in Rhizosperma. In discussions with Dr. Watanabe he had asserted that his priorities in this project were: 1) to end the taxonomic confusion in section Azolla, and 2) to use our techniques for hybrid identification. The first objective was important because of the complete lack of certainty in identifying strains even at the species level in this section. A significant number of accessions in the IRRI collection of these species have dubious and tentative classifications. Agronomically, these species are important since they have shown superiority as biofertilizers in many situations in China and the Philippines compared to the indigenous A. pinnata. The second objective is needed to identify hybrids in the on-going effort to create improved Azolla associations for field application.

Our research has concentrated upon defining the members of section Azolla since that time. The hybrid matter will be discussed first because it is complete. The two strains resulting from hybrid crosses of A. microphylla and A. filiculoides (IRRI nos. 4018 x 1001; F1 4028, 4030) were easily authenticated as hybrids. A third hybrid produced by UP-Los Banos, of which Dr. Watanabe had some question regarding its true nature, was also found to be a probable hybrid during preliminary

experiments conducted during my visit (W. Zimmerman) to IRRI. All hybrid results have been turned over to Dr. Watanabe and will be included in a separate manuscript.

Relative to the first objective, a selected array of accessions from all species of section Azolla-A. filiculoides, A. mexicana, A. caroliniana, A. microphylla, and A. rubra--are presently being evaluated. The work is 90% complete. Cluster analyses on all enzyme results will be realized in mid-February, and first disseminated in March at the 7th International Nitrogen Fixation Congress. A manuscript will be simultaneously prepared.

The enzymic variety within these species, and the apparent mis-identification of several accessions, is illustrated in the accompanying figures. One major personal goal in this matter was to create the simplest possible scheme, using as few as 2 - 4 enzymes, to visually identify the species. This would permit a non-confusing transfer of technology easily utilizable by LDC researchers. Two particularly discerning enzymes are phosphoglucomutase and isocitrate dehydrogenase. From zymograms of only these two enzymes, successful specific identification is already possible for A. mexicana, A. filiculoides, and a subgroup of A. rubra. The other subgroup of A. rubra is identical to A. filiculoides. This classification technique will be perfected and presented together with our statistical analyses in the upcoming manuscript and the next progress report.

The cluster analysis will aid in appropriately and statistically classifying many of the accessions and sub-specific groupings. Some accessions to date, however, have been uniformly identical in all of their phenotypic enzyme banding patterns. We therefore will explore any direct genetic diversity by also analysing DNA restriction fragment length polymorphisms of members of section Azolla. These analyses commence in early April.

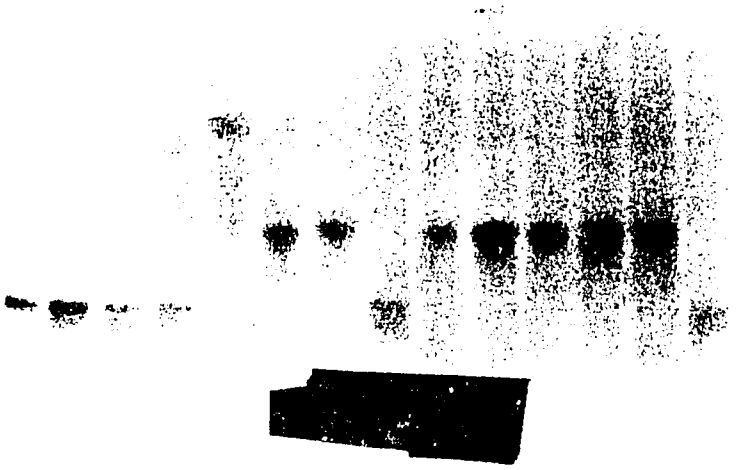
Fig. 1. Isocitrate dehydrogenase zymogram for 15 accessions designated as *Azolla filiculoides*.

Fig. 2. Phosphoglucoisomerase and phosphoglucomutase zymogram for 5 accessions designated as *Azolla mexicana* (lanes 2 - 6) and 8 accessions designated as *Azolla rubra* (lanes 7 - 14). The two outer lanes show the enzymes of *A. filiculoides* IRRI no. 101 as a biological control.

Fig. 3. Phosphoglucoisomerase and phosphoglucomutase zymogram for 14 accessions designated as *Azolla caroliniana*. Lane 1 contains *A. filiculoides* IRRI no. 101.

Fig. 4. Phosphoglucoisomerase and phosphoglucomutase zymogram for 12 accessions designated as *Azolla microphylla*. Lanes 10, 11, and 12 show the enzymes of *A. filiculoides* IRRI no. 101, *A. caroliniana* IRRI no. 301, and *A. rubra* IRRI no. 603, respectively.

Fig. 5. Phosphoglucoisomerase and phosphoglucomutase zymogram of accessions of the five species of section Azolla. IRRI accession numbers (left to right) are: 603, 106, 202, 306, 116, 301, 126, 308, 312, 607, 203, 115, 114, 409.



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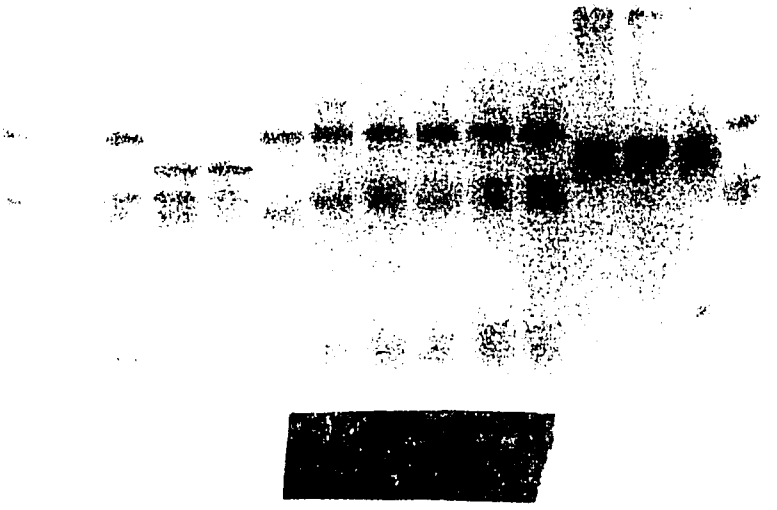

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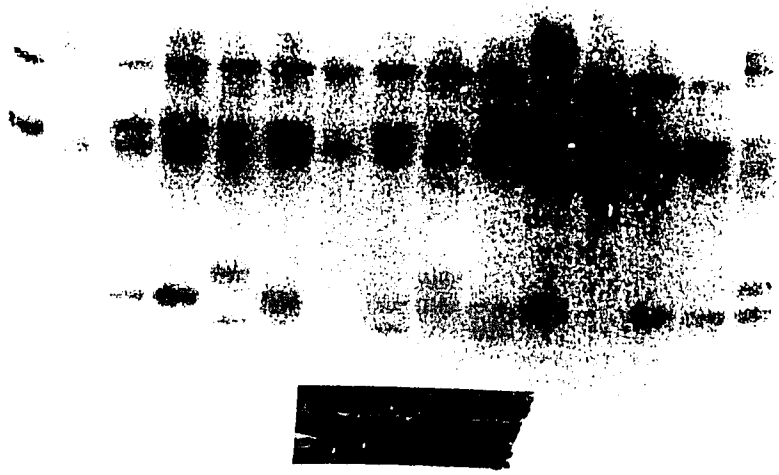

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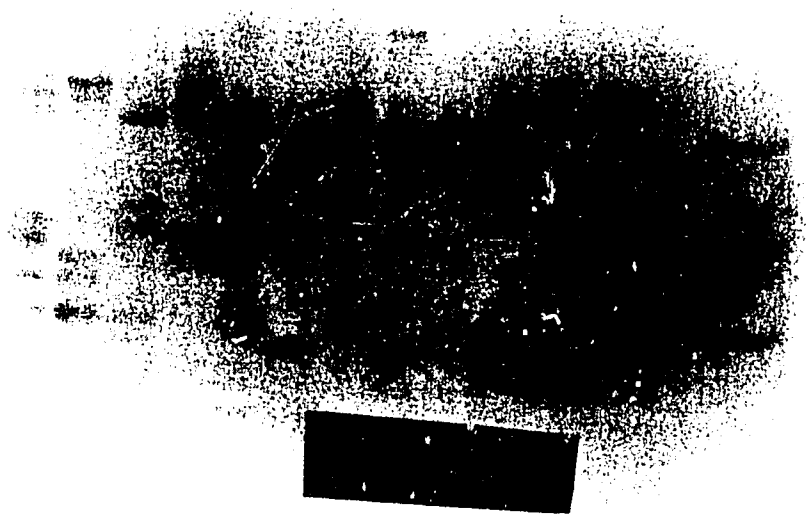

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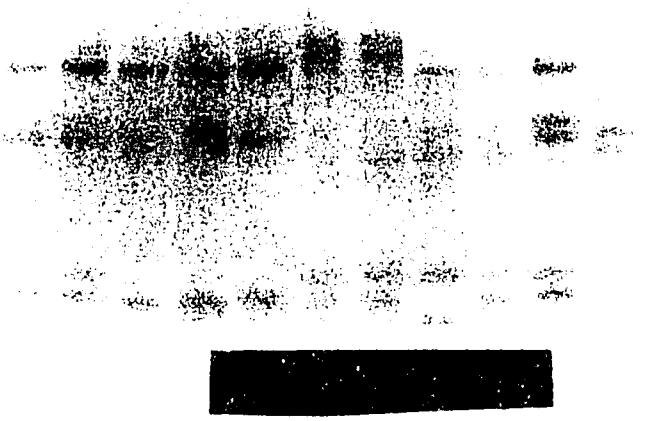

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PROJECT 6446

GRANT NO. DPE-5542-G-SS-6041-00

6.446

to PPC-CDIE

TO: LLOYD

REPORT TO IAEA/FRRI MEETING AT FUZHOU CHINA, OCT 30, 1987.

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FINGERPRINTING OF AZOLLA GERMPLASM

William J. Zimmerman[1], Thomas A. Lumpkin[1], and Iwao Watanabe[2]

[1]Department of Agronomy & Soils, Washington State University, Pullman WA 99164-6420 U.S.A.

[2]Soil Microbiology Unit, International Rice Research Institute, Los Banos, Laguna PHILIPPINES

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ABSTRACT

Progress has been made towards consistently and accurately identifying species and strains of the morphologically plastic aquatic fern Azolla Lam. A standardized biochemical classification of Azolla genepool accessions and a reference for the study of natural populations are the final goals. Objectives of the initial phase were: 1) to optimize methodology for electrophoresis and staining of specific enzymes from Azolla leaf tissue, 2) to provide an overview of the genetic variation among the species, 3) to compare these banding patterns with those of stressed leaf tissue or roots, 4) to observe the differences in the host with and without its endosymbiotic Anabaena azollae, and 5) to illustrate the potential for hybrid identification.

FINGERPRINTING OF *AZOLLA* GERMPLASM

Accurate taxonomic identification is a basic constraint limiting all research with *Azolla* Lam., the aquatic fern which lives symbiotically with the nitrogen-fixing cyanobacterium *Anabaena azollae* Strasb. Using current techniques and information, it is impossible to distinguish varieties of *Azolla* within a species and often difficult to even identify the species. This genus is believed to consist of seven species with classification largely based on the morphology of the reproductive organs. Unfortunately, most accessions of *Azolla* never become fertile under the maintenance conditions of germplasm collections. The identity of any accession may also become suspect after entering a germplasm collection because of the strong possibility of contamination or somaclonal drift over time.

Biochemical systematics is not used for routine classification of these taxa. We have completed the first steps of this process using accessions from the collections of Washington State University and the International Rice Research Institute. Our investigation is a formal collaboration between the two institutes, funded by a grant from the Agency for International Development.

Isozyme (isoenzyme) electrophoresis was selected because of the genetic as well as fingerprinting information that would be accumulated. This methodology is especially valuable for scientists involved in sexually crossing *Azolla* strains or analysing natural populations. The estimation

of genetic diversity is essential in the study of Azolla and also yields practical benefits. Duplicates of genotypically identical accessions, perhaps from redundant collecting of a given area, may be removed, and instability of germplasm during storage may be monitored. Clonal sporophytic tissue from Azolla should normally give identical zymograms, so the documentation of genetic change or the existence of different "electromorphs" (strains or ecotypes) is facilitated.

All accessions of Azolla were cultivated under a moderate, controlled laboratory environment, most in nitrogen-free medium. Samples of the analysed isolates were harvested for electrophoresis before the onset of stationary growth. All species except A. nilotica were included. In most instances, roots were excised (but not rhizomes), leaving predominantly leaf tissue for enzymic analysis. Optimization was completed regarding composition of grinding buffer, PAGE conditions (pH, gel porosity, current, run times), and sample concentration. Supernatants from clarified extracts were stored in liquid nitrogen until the time for electrophoresis.

Enzymes analysed were glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), glutamate dehydrogenase (GDH; EC 1.4.1.2), malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; 1.1.1.40), phosphoglucoisomerase (PGI; EC 5.3.1.9), phosphoglucomutase (PGM; EC 2.7.5.1), glutamine synthase (GS; EC 6.3.1.2), isocitrate dehydrogenase (IDH; EC 1.1.1.42), phosphogluconate dehydrogenase (PGD; EC 1.1.1.44), shikimate dehydrogenase (SkDH; EC 1.1.1.25), and xanthine dehydrogenase (XDH; EC 1.2.1.37). For

species comparison, a simple similarity index was used to compare banding results by their relative anodal migrations.

At least 14 loci were present in this 11-enzyme system. G6PDH, MDH, and SkDH were difficult to interpret, and the first two were the least useful enzymes for obtaining genetic information. Locus 1 of PGI was also not consistently resolvable. Nine strains were tested intensively for illustration of their interspecific differences. Those of section Rhizosperma (A. pinnata var. pinnata and var. imbricata) possessed a similarity index of 38.5% The remaining strains from section Euazolla had a very low similarity of 6.8% since five species were simulataneously compared. Similarities were higher for specific comparisons between A. filiculoides and A. rubra or within the A. caroliniana "group".

Five of the above accessions were also grown in phosphorus-free medium and low temperature (10 ^{o}C) for several weeks to observe the effects of stress on the enzyme banding patterns. The major discernable difference was a much less intense staining of enzymes on the gels. Several enzymes did not yield results for any of the stressed isolates and missing bands were occasionally apparent. Zymograms of the roots of A. pinnata strains were successfully discerned only for G6PDH, ME, PGI, and MDH using our present methodology. Results indicated that root enzymes are not always identical to those of leaf cells. No attempt was made to optimize resolution of enzymes from roots.

Accessions of A. filiculoides with and without a symbiont were compared to observe whether sufficient biomass of Anabaena was present in the association to confer additional bands. The chosen isolates included one direct comparison between a parent strain (IRRI no. 134) and its Anabaena-free cultured derivative (IRRI no. 136). Banding patterns were not identical in these two strains, but differences appear to be from a possible change by the host upon loss of its cyanobiont rather than from a contribution by Anabaena. This conclusion was based on examination of other accessions of A. filiculoides. An example of hybrid identification was obtained by application of several enzyme systems. The tested cross was between A. microphylla and A. filiculoides (IRRI nos. 418 x 101; F1 428,430).

We are currently engaged in the second phase of research with the taxonomy of New World species. Data from the isozymic analyses of numerous accessions will be evaluated in the near future by cluster analysis and dendogram illustration to depict hierarchial relationships. Secondary fingerprinting information from total proteins and DNA will supplement the isozyme results.