Sexual hybridization among *Azolla* species

DO VAN CAT
Faculty of Biology, University of Hanoi, Hanoi, Socialist Republic of Vietnam

IWAO WATANABE
The International Rice Research Institute, Los Baños, Laguna, Philippines

W. J. ZIMMERMAN AND T. A. LUMPKIN
Department of Agronomy and Soils, Washington State University, Pullman, WA 99164, U.S.A.

AND

T. DE WAHA BAILLONVILLE
Laboratory of Plant Physiology, Université Catholique de Louvain, La Neuve, Belgium

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Sexual hybridization, one of possible methods to improve agronomic traits of *Azolla*, was tried among *Azolla filiculoides* Lamarck, *Azolla microphylla* Kaulfuss, *Azolla mexicana* Presl., and *Azolla pinnata* R.Br. Crosses between *A. filiculoides* and *A. microphylla* were successful. Hybrids from *A. microphylla* (female) and *A. filiculoides* (male) were studied in detail. Electrophoresis of enzymes, shikimate dehydrogenase, phosphoglucomutase, and 6-phosphogluconate dehydrogenase, showed that enzyme loci of hybrids shared those of one or both parents. Hybrids produced only microsporocarps. Stem length of the hybrid was intermediate between those of both parents. *Azolla filiculoides* has longer stems than *A. microphylla*. Red color appeared in hybrids but not in both parents in the field and in the greenhouse under the P- or Ca-deficient condition. Fresh biomass production of hybrids at 37°C: 29°C 12-h light : 12-H dark was lower than that of *A. microphylla* and higher than that of *A. filiculoides*. The N content of hybrids was higher than that of *A. microphylla*. Growth of hybrids in the field was greater than that of *A. microphylla* strains, indicating positive heterosis.

Key words: *Azolla*, *Azolla filiculoides*, *Azolla microphylla*, sexual hybridization, heterosis.

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L'hybridization sexuelle, une des methodes potentielles pour l'amélioration des caractéristiques agronomiques d'*Azolla*, a été testée sur des souches d'*Azolla filiculoides* Lamarck, *Azolla microphylla* Kaulfuss, *Azolla mexicana* Presl. et *Azolla pinnata* R.Br. Les croisements entre *A. filiculoides* et *A. microphylla* ont produit des hybrides. Ceux ayant *A. microphylla* comme parent femelle ont pu être étudié en détail. L'étude des zymogrammes de la shikimate déhydrogenase, de la phosphoglucomutase, et de la 6-phosphogluconate déhydrogenase montre que les loci des enzymes des hybrides sont communs a l'un des parents ou aux deux parents. Les hybrides ont produit uniquement des microsporocarpes. La longueur de la tige des hybrides est intermédiaire entre celle d'*A. filiculoides* (tige longue) et celle d'*A. microphylla* (tige courte). Les hybrides se développant *in situ* ou en serre présentent une coloration rouge lorsque le milieu est déficient en phosphore ou en calcium, alors que les parents ne présentent pas cette caractéristique. La productivité *in vitro* des hybrides (37°C durant le jour (12 h) et 29°C pendant la nuit) est inférieure a celle d'*A. microphylla* et supérieure a celle d'*A. filiculoides*. La teneur en azote des hybrides est supérieure a celle d' *A. microphylla*. Une productivité *in situ* supérieure chez les hybrides a celle de diverses souches d'*A. microphylla* indique un hétérosis positif.

Mots clés : *Azolla*, *Azolla filiculoides*, *Azolla microphylla*, hybridization sexuelle, hétérosis.

Introduction

Azolla has long been utilized in China and northern Vietnam as green manure for wetland rice (Lumpkin and Plucknett 1982). Recently, its use in the Philippines was reported (Kikuchi et al. 1984). The wider utilization of *Azolla* for agricultural purposes is limited by various biological constraints: low tolerance for high temperature and insect damage, high requirement of phosphorus (Watanabe 1986), high lignin content and low digestibility (Scharpenseel and Knuth 1987), and low amounts of some essential amino acids (Van Hove et al. 1987). *Azolla filiculoides* introduced in China in the 1970s had difficulties in growth during summer owing to its low tolerance for high temperature (Li 1984).

Screening from culture collections has been the only way to obtain *Azolla* strains with favorable agronomic traits. Without sexual recombination of genomes, improvement of *Azolla* germ plasm is difficult. Successes in sexual hybridization among different species of Azolla have been reported in China (cited by Wei et al. 1986). Wei et al. (1986) reported sexual hybridization between *Azolla microphylla* and *A. filiculoides*. Both species were used as male or female parents. Hybridization was confirmed by the use of zymograms of fern and monoclonal antibodies against *Anabaena azollae*.

The paper reports sexual hybridization trials among four *Azolla* species and describes some characteristics of the hybrids obtained.

Materials and methods

Strains of Azolla *used*

Azolla microphylla Kaulfuss from Paraguay (International Rice Research Institute (IRRI) accession No. 4018), *A. filiculoides* Lamarck from West Germany (accession No. 1001), *Azolla pinnata* var. *pinnata* R.Br. from the Northern Territory of Australia (accession No. 7001), and *Azolla mexicana* Presl. from U.S.A. (accession No. 2001) were used for crosses.

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Collection of sporocarps

All strains grown in the IRRI paddy field produced megasporocarps and microsporocarps. The formation and germination of sporocarps were most active during the dry, cooler season. Mature sporocarps are brown with dark brown tips. These were collected from the plants. The megasporocarps and microsporocarps collected were stored separately in the dark at room temperature but were used within a month for germination.

Hybridization and germination

Megasporocarps free from attached massulae and microsporocarps of different species were mixed in distilled water at a ratio of one megasporocarp to ten microsporocarps. The mixture was placed under weak light (1.6 W/m^2) at room temperature (25−30°C). When plants had three or five leaves, the new sporophytes were grown in N containing the IRRI water culture medium (Watanabe et al. 1977). These sporophytes were kept in a Phytotron at 50 W/m^2 and 24°C in culture solutions that either contained or did not contain N.

Zymograms

Zymograms of hybrids and their parents grown in the IRRI water culture were made using the method described by Zimmerman et al. (1989). Extraction of water-soluble enzymes was made after removing the bulk of the root. Shikimate dehydrogenase (SKDH; EC 1.1.1.5), phosphoglucomutase (PGM; EC 2.7.5.1), and 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44) were used. For 6PGD, 7.5% polyacrylamide gel was used; for other enzymes, 10% was used.

Growth in controlled conditions

Azolla strains were grown in water culture medium at 26°C:18°C 12-h light : 12-h dark in a Koitotron KG cabinet (Koito Kogyo Co., Tokyo) with 50 W/m^2 light intensity and 75% minimum relative humidity. Plant density was maintained at full cover with frequent removel of excess biomass. After 1 week of growth, 0.7 g fresh weight (95 g/m^2) was inoculated into a bottle, 10 cm in diameter, containing 450 mL mineral medium. Temperature was set at 37°C: 29°C light−dark. The other conditions were the same as those during pretreatment. The plants were grown for 4 weeks, with biomass determination made every week. When *Azolla* fully covered the water surface, biomass density was thinned to 0.7 g fresh weight. Acetylene reduction activity was measured for the *Azolla* removed (Tung and Watanabe 1983).

Field growth

The hybrids and parents were grown outside in concrete pots with rice soil at IRRI. To compare biomass production, 40 *Azolla* strains, including hybrids 4028 and 4030 and *A. microphylla* 4018, were grown in an IRRI flooded rice field with weekly application of phosphate fertilizer (5 kg P_2O_5/ha). *Azollla filiculoides* could not give sufficient biomass to conduct experiments in the field. *Azolla* was inoculated every week at 1 kg fresh weight/m^2 in a 0.5-m^2 round floating basket. After 1 week, the basket was removed from the field, drained for 10 min, and weighed. Two replicates were set for each strain. Details of determining biomass production by using a floating basket will be reported separately.

Results

Formation of F_1

When microsporocarps and megasporocarps of species belonging to subgenus *Azolla* were mixed, massulae released from microsporangia anchored on the perispore surface of the megasporocarps, and megasporocarps were entangled through the bridge of massulae. When microsporocarps of *A. pinnata* mixed with megasporocarps of *A. microphylla* or *A. filiculoides*, the massulae combined with megasporocarps so weakly that massulae were separated from the megasporocarps.

Crosses between *A. filiculoides* (female) and *A. microphylla* (male) and between *A. microphylla* (female) and *A. filiculoides* (male) were successful. Out of 750 crosses between *A. microphylla* (female) and *A. filiculoides*, 130 sporocarps germinated, but only 29 showed embryo development. It took 13 days before the cotyledon became visible. Among them, eight were albino and eventually died. Most of the green sporophytes also died during maintenance at IRRI, and eventually only three strains, 4028, 4029, and 4030, survived. Strain 4029 is *Anabaena*-free.

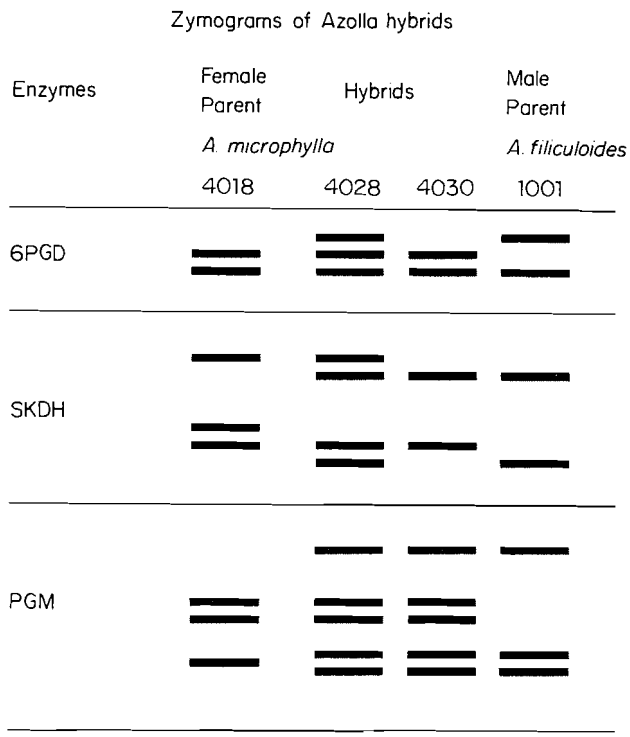

FIG. 1. Zymograms of parents and hybrids. Upper portion is directed to cathode.

Out of 560 crosses between *A. filiculoides* (female) and *A. microphylla*, 75 germinated but only 14 showed embryo development. It took 10 days before the cotyledon became visible. Six plants were albinos. Eventually only one *Anabaena*-free strain, 1033, survived. Symbiotic *Azolla* from *A. microphylla* and *A. filiculoides* (4028 and 4030) were, therefore, studied in detail and propagated for growing in the field.

Zymograms

Enzymes of *Anabaena* were not extracted by the gentle water extraction used here (Zimmerman et al. 1989). The electrophoresis patterns of three enzymes (6PGD, PGM, and SKDH) are shown in Fig. 1. In hybrids, bands specific to each parent were found in one or both of the hybrids. It is, therefore, concluded that strains 4028 and 4030 are hybrids between *A. filiculoides* and *A. microphylla* because of enzyme band complementarity.

Morphological characteristics

The hybrids produced red anthocyanin pigments in the field and greenhouse under P- or Ca-deficient conditions in water culture, whereas both parents did not. Hybrids did not produce megasporocarps but only microsporocarps. The number of septa in glochidia was 1 or 0, a characteristic property of *A. filiculoides*. Trichomes on dorsal lobes had one pedical cell

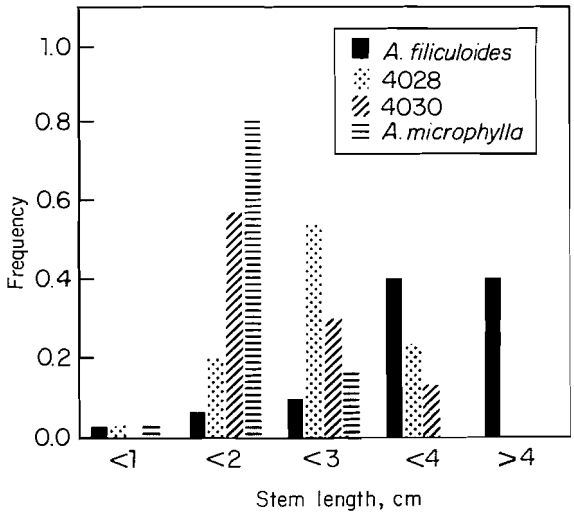

FIG. 2. The distribution of stem length in parents and hybrids.

and one or two apical cells, a characteristic property of *A. microphylla*.

Only in December–February, when temperature is lowest in the IRRI field, did *A. filiculoides* grow well and produce elongated stems. Distribution (30 plants) of the length of stems (rhizomes) in *Azolla* grown in the field in January showed the intermediate character of hybrids (Fig. 2). The average stem length (in cm) was 3.7 (±0.2 SE) in *A. filiculoides*, 1.6 (±0.08) in *A. microphylla*, 2.5 (±0.2) in hybrid 4028, and 2.0 (±0.2) in hybrid 4030. The differences between hybrids and parents are significant.

Physiological characters

Azolla filiculoides is more sensitive to high temperature than *A. microphylla* (Tung and Watanabe 1983). As shown in Fig. 3, *A. filiculoides* stopped growth after 1 week at 37°C:29°C. At 28 days, the hybrids produced larger biomass than *A. filiculoides* did. On the other hand, the hybrids produced 20–28% less biomass than *A. microphylla*. This slightly lower biomass production of hybrids is statistically significant (SE, 37 g/m^2).

Acetylene reduction activities (ARA) of both hybrid strains were not different from those of *A. microphylla*. Nitrogen on a dry weight basis at 28 days was 2.4% in *A. filiculoides*, 3.57% in *A. microphylla*, 3.91% in hybrid 4028, and 4.09% in hybrid 4030. The differences in percent N between *A. microphylla* and hybrids are significant (SE, 0.11). Similar results were obtained in another trial. Fresh biomass production of hybrids at 28 days was 20–16% lower (statistically significant) than that of *A. microphylla*. Nitrogen was 1.50% in *A. filiculoides*, 3.78% in *A. microphylla*, 4.78% in hybrid 4028, and 4.97% in hybrid 4030. The hybrids had higher percent N (SE, 0.10) than *A. microphylla*, suggesting higher nitrogen-fixing activity, although ARA was not different as a result of a large variability in ARA measurement.

Growth in the field

Field trials during December and February showed that both hybrids produced significantly high biomass (average of 129 g fresh weight/(m^2 · d) than did parent *A. microphylla* (average of 89 g fresh weight/(m^2 · d). Another *A. microphylla* 4509 produced an average of 79 g fresh weight/(m^2 · d) (Fig. 4). These data suggest a positive heterosis in the hybrids.

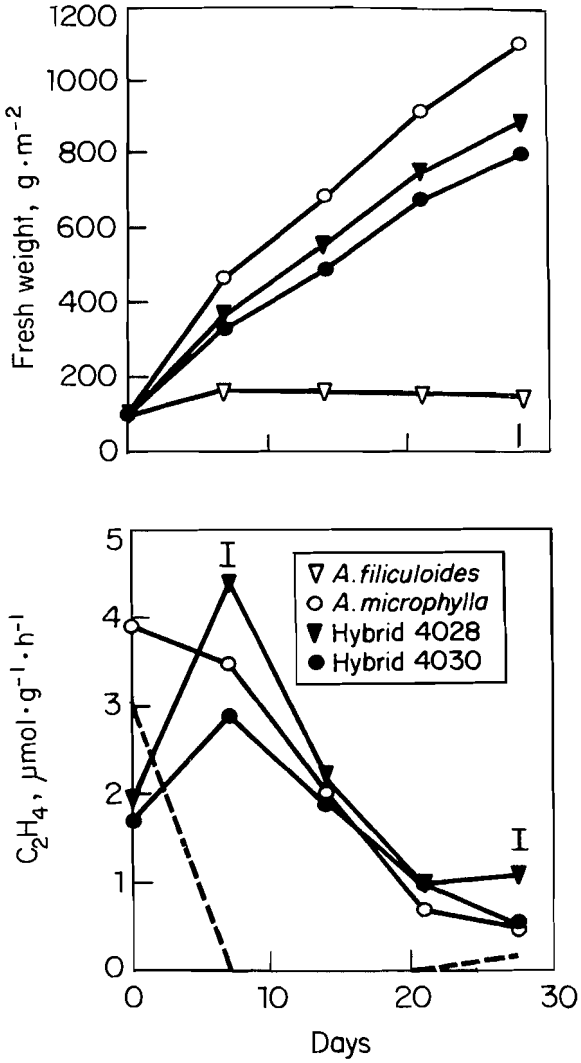

FIG. 3. Fresh biomass and acetylene reduction activity of parents and hybrids. Vertical bars indicate standard errors. Acetylene reduction activity of *Azolla filiculoides* was not measured at 1, 2, and 3 weeks.

Discussion

To avoid self-fertilization, hybridization was made between microsporocarps and megasporocarps collected separately from the parent fern. Wei et al. (1986) either manually or chemically removed massulae attached to the megasporocarps and reported lower germination rates after chemical emasculation. Hybridization of *A. microphylla* with *A. filiculoides* was confirmed by the inheritance of enzyme loci from both parents. Morphological characteristics also indicated hybridization. Both parents do not produce anthocyanin pigments, but the hybrids produced them in the field and in the greenhouse under P- or Ca-deficient conditions. Low tolerance of *A. filiculoides* for high temperature did not appear in the hybrids. The hybrids showed positive heterosis or hybrid vigor in the growth in the field and nitrogen fixation at 33°C. Because *Anabaena* is transferred from the megasporocarps to the new sporophyte, *Anabaena* in the hybrid came from *A. microphylla*. Watanabe et al. (1989) showed that *Anabaena* cells from *A. microphylla* were more tolerant of high temperature than those from *A. filiculoides*. If *Anabaena* primarily deter-

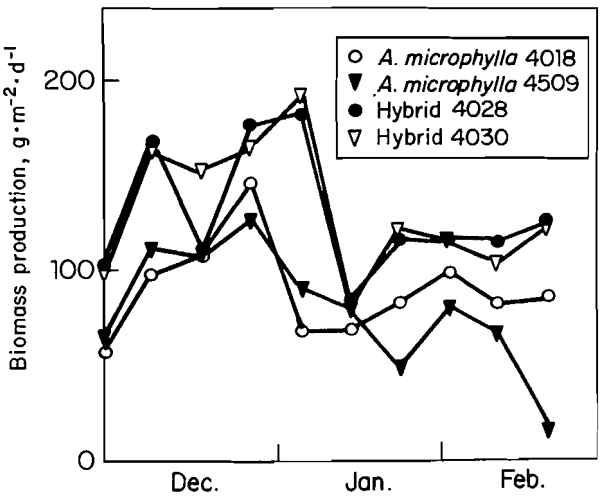

FIG. 4. Daily fresh biomass production of hybrids and two strains of *Azolla microphylla* in the field from 8 December to 16 February. Average standard error is 0.12 $g \cdot m^{-2} \cdot d^{-1}$.

mines tolerance for high temperature, the maintenance of temperature tolerance in the hybrid is understandable. The response of the hybrids to low temperature was not tested.

The rate of hybridization of *A. filiculoides* with microsporocarps of *A. microphylla* was less than those of *A. microphylla* with *A. filiculoides*. A similar observation was made by Wei et al. (1986), who noted a higher frequency of albinos in the hybridized sporophyte when *A. filiculoides* was the female parent. Because the hybrids did not produce megasporocarps, segregation in the F_2 was not confirmed. Wei et al. (1986) also did not observe megasporocarps in their hybrids of *A. microphylla* with *A. filiculoides* that were morphologically similar to ours. They also did not succeed in crossing *Rhizosperma* species with *(Eu)azolla* species.

Owing to its elongated stems, *A. filiculoides* can produce higher biomass in optimum conditions than can *A. microphylla*. It is, however, sensitive to high temperature. Crossing *A. microphylla* with *A. filiculoides* may be a way to enhance the genetic potential of *Azolla*. Positive heterosis exemplified in the hybrids between *A. microphylla* and *A. filiculoides* may give promise in improving *Azolla–Anabaena* symbiosis.

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