

Clonal reproduction of *Azolla filiculoides* Lam.: implications for invasiveness

Rocío Fernández-Zamudio^{1,*}, Santos Cirujano², Salvador Sánchez-Carrillo³, Ana Meco² and Pablo García-Murillo¹

¹ Department of Plant Biology and Ecology, Faculty of Pharmacy, University of Seville, C/ Profesor García González, 2, 41012 Seville, Spain.

² Royal Botanical Garden, Madrid (CSIC), Plaza de Murillo 2, 28014 Madrid, Spain.

³ National Museum of Natural Sciences (MNCN-CSIC), C/ José Gutiérrez Abascal 2 28006 Madrid, Spain.

* Corresponding author: rzamudio@us.es

Received: 30/10/12

Accepted: 24/7/13

ABSTRACT

Clonal reproduction of *Azolla filiculoides* Lam.: implications for invasiveness

Clonal reproduction of *Azolla filiculoides* Lam., an aquatic floating fern, native from the American subtropics and a recent invader of Mediterranean wetlands, was assessed experimentally. Shoot fragmentation of the species was quantified under suitable room conditions for optimum growth. *Azolla filiculoides* showed high longevity, a low mortality percentage and a high potential for clonal reproduction (biomass obtained by shoot fragmentation from only one individual was multiplied by 100). This last attribute changed over time: first generations developed a high number of propagules, a number which decreased progressively in later generations until the end of the experiment. The results of this experiment will be useful for understanding *Azolla*'s success within invaded habitats.

Key words: *Azolla*, plant clonal reproduction, invasive species, Iberian Peninsula.

RESUMEN

Reproducción clonal de *Azolla filiculoides* Lam.: consecuencias para su éxito invasor

La reproducción vegetativa por fragmentación de *Azolla filiculoides*, helecho acuático propio de medios subtropicales americanos pero presente recientemente en medios acuáticos temporales, fue valorada experimentalmente. Se cuantificó la tasa de fragmentación de la especie en condiciones controladas y favorables para asegurar un crecimiento óptimo de la especie. *Azolla filiculoides* presentó una alta longevidad, una baja tasa de mortalidad y una alta capacidad de reproducción vegetativa por fragmentación (la biomasa obtenida a partir de un solo individuo se multiplicó por 100 durante la experiencia). Sin embargo la tasa de fragmentación no fue constante: las primeras generaciones generaron un número superior de propágulos que fue decreciendo exponencialmente en generaciones posteriores hasta el final de la experiencia. Los resultados obtenidos resultan útiles para entender el éxito de la colonización en medios invadidos.

Palabras clave: *Azolla*, reproducción clonal, especies invasoras, Península Ibérica.

INTRODUCTION

Perennial plants usually combine sexual reproduction by seeding with clonal reproduction

through vegetative propagation (Eckert *et al.*, 2003). In most aquatic angiosperm taxa, asexual modes of reproduction are dominant over sexual modes (Barret *et al.*, 1993; Grace, 1993; Garbey

et al., 2004; Xiao *et al.*, 2007). Asexual modes are usually induced by adverse environmental conditions or by a lack of resource availability, inhibiting normal life cycle (Barret *et al.*, 1993; Honnay & Bossuyt, 2005). Among invasive species, almost 60 % are clonal, which suggests that clonal growth may be associated with invasiveness (Alpert *et al.*, 2000; Kolar & Lodge, 2001). In this context, vegetative propagation is not only understood as a way of ensuring the survival of populations but also as a great opportunity for rapid and efficient dispersion and new habitat colonisation (Sculthorpe, 1967).

One of the most common methods of clonal reproduction in submerged and free-floating flora is shoot fragmentation (Wetzel, 2005). Detaching fragments function as new individuals with the ability to photosynthesise, uptake nutrients from the water column, multiply, and disperse using water as an efficient vector (Grace, 1993).

Azolla filiculoides Lam. (Azollaceae, hereafter AF) is a small floating fern and native from the American subtropics (Landolt, 1999; Galán de Mera *et al.*, 2006). It is broadly distributed in

aquatic ecosystems all over the world and is considered in some parts to be an invasive species (Sheppard *et al.*, 2006). It reproduces asexually via shoot fragmentation, an ability that ensures a high increase of populations in the absence of environmental constraints (Wagner, 1997).

The aim of this work is to assess the clonal reproduction model of AF, by studying shoot fragmentation rates of individuals growing in the absence of environmental constraints. For this purpose, individuals of AF were obtained from spores found in Doñana National Park (SO Spain). This area, a Ramsar wetland well-known for its conservation value (García-Novo & Marín, 2006), has been recently colonised by AF (García-Murillo *et al.*, 2007).

MATERIAL AND METHODS

Experimental design

Owing to the impossibility of running this experiment in the field, we decided to work under labo-

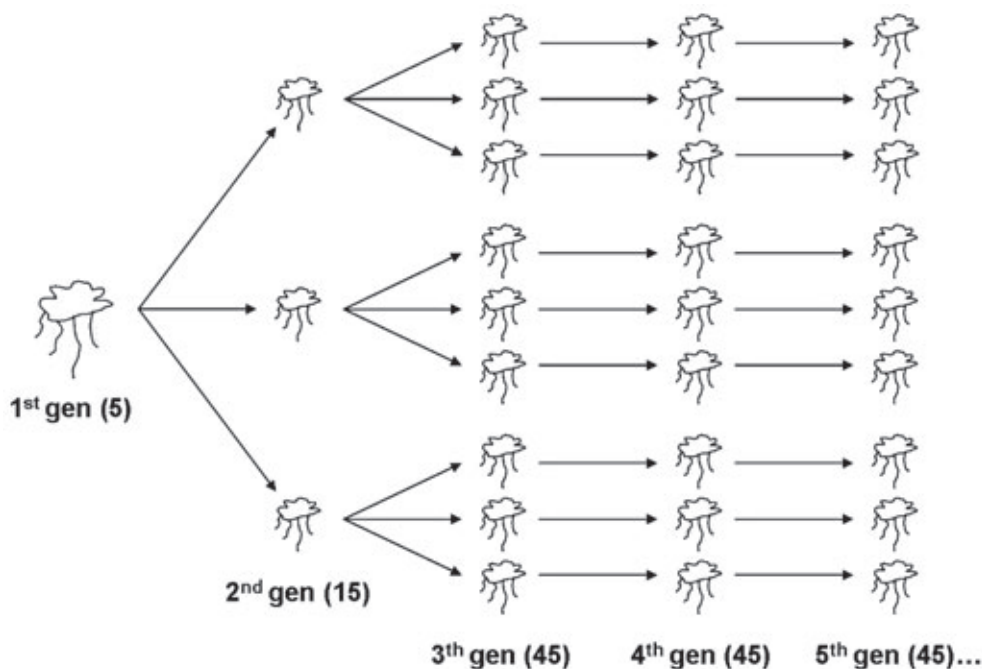


Figure 1. Selection procedure of propagules included in the experimental design from mother-plants. *Resumen de la selección de propágulos a partir de las planta-madre para ser incluidos en el diseño experimental.*

ratory conditions, similarly to how other physiological experiments with *Azolla* (Cary & Weerts, 1992; Janes *et al.*, 1996; Rai *et al.*, 2006) have been developed.

We chose five just-germinated individuals of AF (hereafter referred to as mother-plants) which were measured and weighed before the beginning of the experiment. The individuals were placed in separate holes in plastic seedbeds under greenhouse conditions [regime of 12 h of light (mean maximum temperature 37.6 °C, 400-700 nm, 35 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) and 12 h of darkness (mean minimum temperature 17.9 °C)] to ensure optimum growth. The individuals were submerged in trays filled with 1 cm of standard compost (sand+peat+humus) and 10 l of water (salinity in a range of 700-1000 $\mu\text{S cm}^{-1}$ and pH of approximately 7.5). Water was supplemented with sodium monophosphate to reach an optimal concentration of dissolved phosphorus (1.5 mg l^{-1}) for the AF survival (Cirujano *et al.*, 2008). Culture media was replaced every week to ensure phosphorus availability.

From each of the initial five mother-plants, three new propagules obtained via shoot fragmentation were selected to be incorporated into the experiment (second generation). For the third generation, three new propagules were selected from each of the fifteen individuals, resulting in sixty individuals placed in the fourth generation; from the 4th through the 11th generations, only one propagule from each individual was selected and incorporated into the experiment (Fig. 1). The remaining individuals of every generation were discarded after being counted.

In the end, the shoot fragmentation of 324 individuals were followed weekly over 215 days, to calculate the total number of propagules per generation, the maximum number of propagules per individual, the fragmentation rate (number of propagules per day) and the mortality rate (number of dead individuals per day).

Data analyses

The differences between generations in the total number of propagules, the fragmentation rate and the maximum number of propagules per in-

dividual, were analysed through one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. The significance level (p) considered throughout the experiment was 0.05 and the analyses were carried out with the software Statistica v.6 (Statsoft, 2001).

The increase in biomass through time was estimated from the initial biomass values measured from the mother-plants and the fragmentation rate data obtained from the experiment. The results were fitted to a non-linear (sigmoidal) function that permitted us to assess the potential biomass per capita rate over time. Curve fitting was carried out with CurveExpert v.1.4.

RESULTS

The mean initial length of the mother-plants was 7 mm (length) \times 2 mm (width), with an initial fresh biomass of 0.0044 ± 0.0025 g (mean \pm SE). The total number of propagules per generation was 15.05 ± 2.18 (mean \pm SE). None of the mother-plants died during the experiment; however, some of the individuals produced by shoot fragmentation died. The mortality rate for these individuals was 1.60 ± 0.71 % (mean \pm SE). There were no sexual structures observed during the experiment.

The total number of propagules per generation showed significant differences between generations ($F_{10,313} = 34.116$; $p = 0.0001$). First generations developed a high number of propagules, a number which decreased progressively in later generations [45.60 ± 11.19 and 0.50 ± 0.27 in the 1st and 11th generation, respectively (Table 1)]. The maximum number of propagules per individual followed the same pattern [$(F_{10,313} = 21.954$; $p = 0.000)$, 7.80 ± 1.36 and 0.40 ± 0.22 in 1st and 11th generation, respectively (Table 1)]. The fragmentation rate also varied significantly between generations ($F_{10,313} = 34.116$; $p = 0.0001$), decreasing exponentially (Fig. 2).

The temporal increase in biomass was fitted to a sigmoidal function ($r = 0.998$; SE = 0.01, Fig. 3). Applying this function, an individual with an initial biomass of 0.0044 ± 0.0025 g ($n = 5$), would through shoot fragmentation and

Table 1. Mean number of propagules, maximum number of propagules and fragmentation rate of *Azolla filiculoides* between generations (mean \pm SE). *Evolución temporal del número medio de vástagos producidos por plantas-madre de Azolla filiculoides representativas de cada generación (media \pm SE).*

Generation (N)	Total Mean N° Propagules	Fragmentation Rate	Maximum N° Propagules/ind
1 (5)	45.60 \pm 11.19	0.21 \pm 0.05	7.80 \pm 1.36
2 (15)	33.47 \pm 4.16	0.16 \pm 0.02	6.60 \pm 0.94
3 (45)	21.84 \pm 1.72	0.10 \pm 0.01	4.49 \pm 0.41
4 (45)	17.40 \pm 1.28	0.08 \pm 0.01	3.84 \pm 0.31
5 (45)	13.93 \pm 1.18	0.06 \pm 0.01	3.04 \pm 0.25
6 (43)	11.06 \pm 1.01	0.05 \pm 0.00	2.72 \pm 0.25
7 (39)	11.05 \pm 1.06	0.05 \pm 0.00	2.51 \pm 0.19
8 (24)	6.18 \pm 1.01	0.03 \pm 0.00	1.76 \pm 0.22
9 (27)	2.63 \pm 0.57	0.01 \pm 0.00	1.07 \pm 0.21
10 (16)	1.87 \pm 0.57	0.01 \pm 0.00	1.12 \pm 0.24
11 (10)	0.50 \pm 0.27	0.00 \pm 0.00	0.40 \pm 0.22

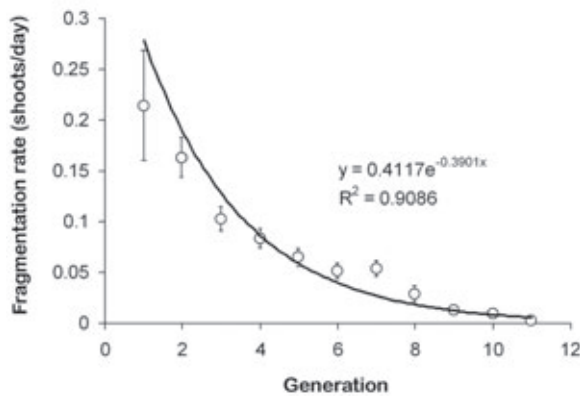


Figure 2. Mean \pm SE values for the fragmentation rate as fitted along generations of *Azolla filiculoides* ($p < 0.05$). *Ajuste de los valores medios \pm SE de las tasas de fragmentación obtenidas de los individuos de Azolla filiculoides representativos de cada generación.*

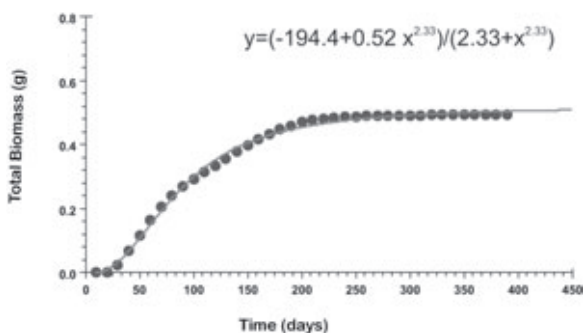


Figure 3. Model fitted to the increase of biomass of *Azolla filiculoides* through time. *Modelo ajustado al incremento en biomasa de Azolla filiculoides a lo largo del tiempo.*

under unconstrained environmental conditions, produces new individuals with a final biomass of 0.4990 g in one year.

DISCUSSION

AF is recognised as an invasive alien plant (Shepard *et al.*, 2006) and fragmentation is the most likely cause of its explosive spread (Sculthorpe, 1967; Wagner, 1997).

The results obtained from the sigmoidal function model demonstrate that AF shows a high potential for clonal reproduction in the absence of environmental constraints (the final biomass obtained by shoot fragmentation is multiplied by 100 in one year). The large number of propagules obtained by shoot fragmentation enhances dispersion (Grace, 1993), facilitates the colonisation of new areas and maximises gene flow between populations; thus, providing enough genetic variability between localities (Barret *et al.*, 1993).

According to others authors, floating macrophytes (Lemnaceae: *Lemna* spp., *Spirodella* spp., *Wolffia* spp., Table 2) have a lifespan of approximately 30 days (Ashby *et al.*, 1949; Lemon *et al.*, 2001), which is much lower than the lifespan observed for AF [initial individuals stayed alive throughout the experiment (215 days) and the mortality rate of propagules was very low (almost 1.60 \pm 0.71 % of the total number of propagules produced)]. Lemon *et al.*, (2001) found the mul-

Table 2. Summary of values for the lifespan, number of propagules and fragmentation rate of different floating macrophytes. *Resumen de diferentes valores de esperanza de vida, numero de propágulos producidos y tasa de fragmentación para diferentes especies de macrófitos flotantes.*

Especies	Lifespan (days)	Nº Propagules	Fragmentation Rate	Reference
<i>Salvinia auriculata</i>	—	46 to 281	—	Coelho <i>et al.</i> , 2005
<i>Wolffia borealis</i>	15.8 ± 1.5	9.8 ± 0.7	0.62 ± 0.03	Lemon <i>et al.</i> , 2001
<i>Lemna minor</i>	31.3 ± 1.1	14.0 ± 0.5	0.45 ± 0.02	Lemon <i>et al.</i> , 2001
<i>Sphyrrodela polyrhiza</i>	12.1 ± 1.1	1.1 ± 0.5	0.08 ± 0.02	Lemon <i>et al.</i> , 2001
<i>Lemna minor</i>	35 to 42	—	—	Ashby <i>et al.</i> , 1949

tipling strategy of macrophytes to be influenced by the life expectancy of mother-plants and an individual's potential to make propagules. Accordingly, our results are useful in understanding *Azolla's* life history strategy. The high longevity of mother-plants, together with the species' high ability to produce propagules by shoot fragmentation, represents an adaptive advantage that facilitates the success of colonisation by clonal reproduction.

However, in the absence of environmental constraints, the total and maximum number of propagules and the fragmentation rate varied through time. At the start of the experiment, we obtained a high number of propagules, but this number decreased progressively until the end of the experiment. These results are related to the aging of individual's within the population through time, as well as the effect clonal reproduction has on a population's dynamics. Accordingly, high levels of clonal recruitment in populations, where sexual recruitment is inhibited, are expected to strongly influence genetic variation within and among populations (Eckert, 2002). In this situation, individuals would lose the adaptive capability to face environmental variations (Eckert & Barret, 1992).

Finally, the *Azolla* individuals used in our experiment came from Doñana temporary wetlands. This place represents a "new habitat" with environmental features quite different than *Azolla's* native ecosystems (Galán de Mera *et al.*, 2006). The most apparent difference is the existence of a summer drought period each year (Díaz-Paniagua *et al.*, 2010). Such characteristics could enhance the presence of specific ecotypes adapted and restricted to the environmental characteristics of the Doñana ecosystem. In this adaptation process, the symbiotic prokaryotic

Azolla's community plays a key role when facing new environmental stress conditions (Carrapiço, 2002, 2010). Sexual reproduction seems to be the best option to ensure a population's resistance after drought periods. Thus, further research on the *Azolla-anabaena* system and the sexual reproductive strategies of *Azolla filiculoides* in temporary habitats should be developed.

ACKNOWLEDGEMENTS

We are grateful to Laura Gonzalez-Guzman from the University of Texas at Austin for reviewing the English language and syntax.

This study was funded by the Spanish Ministry of Science and Education and EU FEDER funds (CGL2009-09801), the National Park Funding and the Spanish Ministry of Environment, Agriculture and Marine (158/2010). R.F.Z. holds a fellowship grant from the Andalusian Regional Government (2003).

REFERENCES

- ALPERT, P., E. BONE & C. HOLZAPFEL. 2000. Invasiveness, invasibility and the role of environmental stress in the spread of non-native plants. *Perspectives in Plant Ecology, Evolution and Systematics*, 3: 52–66.
- ASHBY, E., E. WANGERMAN & E. J. WINTER. 1949. Studies in the morphogenesis of leaves III. Preliminary observations on vegetative growth in *Lemna minor*. *New Phytologist*, 48: 374–381.
- BARRET, S. C. H., C. G. ECKERT & B. C. HUSBAND. 1993. Evolutionary processes in aquatic plant populations. *Aquatic Botany*, 44: 105–145.

- CARRAPIÇO, F. 2002. The *Azolla-Anabaena*-Bacteria system as a natural microcosm. *Proceedings of SPIEF* 4495: 261–265.
- CARRAPIÇO, F. 2010. *Azolla* as superorganism. Its implication in symbiotic studies. *Celular Origin, Life in Extreme Habitats and Astrobiology* 17: 225–241.
- CARY, P. R. & P. G. J. WEERTS. 1992. Growth and nutrient composition of *Azolla pinnata* R. Brown and *Azolla filiculoides* Lammark as affected by water temperature, nitrogen and phosphorus supply, light intensity and pH. *Aquatic Botany*, 43: 163–180.
- CIRUJANO, S., P. GARCÍA-MURILLO, R. FERNÁNDEZ-ZAMUDIO, J. M. ESPINAR, A. RUBIO, A. MECO, I. LÓPEZ-BRAVO, R. SÁNCHEZ-ANDRÉS, S. SÁNCHEZ-CARRILLO, M. MORENO & A. SOUSA. 2008. *Estudio y control del helecho acuático invasor Azolla filiculoides en los humedales del Parque Nacional de Doñana 2005-2008* (Technical report). Royal Botanic Garden of Madrid (CSIC). Madrid, Spain.
- COELHO, F. F., LOPES, F. S., SPERBER, C. F. 2005. Persistence strategy of *Salvinia auriculata* Aublet in temporary ponds of Southern Pantanal, Brazil. *Aquatic Botany* 81: 343–352.
- DÍAZ-PANIAGUA, C., R. FERNÁNDEZ-ZAMUDIO, M. FLORENCIO, P. GARCÍA-MURILLO, C. GÓMEZ-RODRÍGUEZ, A. PORTHEAULT, L. SERRANO & P. SILJESTROM. 2010. Temporary ponds from Doñana National Park: a system of natural habitats for the preservation of aquatic flora and fauna. *Limnetica*, 29: 41–58.
- ECKERT, C. G. 2002. The loss of sex in clonal plants. *Evolutionary Ecology*, 15: 501–520.
- ECKERT, C. G. & S. C. H. BARRETT. 1992. Stochastic loss of style morphs from populations of tristylous *Lythrum salicaria* and *Decodon verticillatus* (Lythraceae). *Evolution*, 46: 1014–1029.
- ECKERT, C. G., K. LUI, K. BRONSON, P. CORRADINI & A. BRUNEAU. 2003. Population genetic consequences of extreme variation in sexual and clonal reproduction in an aquatic plant. *Molecular Ecology*, 12: 331–344.
- GALÁN DE MERA, A., A. GONZÁLEZ, R. MORALES, B. OLTRA & J. A. VICENTE ORELLANA. 2006. Datos sobre la vegetación de los llanos occidentales del Orinoco (Venezuela). *Acta Botánica Malacitana*, 31: 97–129.
- GARBAY, C., G. THIÉBAUT & S. MULLER. 2004. Morphological plasticity of a spreading aquatic macrophyte, *Ranunculus peltatus*, in response to environmental variables. *Plant Ecology*, 173: 125–137.
- GARCÍA-MURILLO, P., R. FERNÁNDEZ-ZAMUDIO, S. CIRUJANO, A. SOUSA & J. M. ESPINAR. 2007. The invasion of Doñana National Park (SW Spain) by the mosquito fern (*Azolla filiculoides* Lam). *Limnetica*, 26: 243–250.
- GARCÍA-NOVO, F. & C. MARÍN. (EDS.). 2006. *Doñana. Water and biosphere*. Confederación Hidrográfica del Guadalquivir y Ministerio de Medio Ambiente. Madrid. Spain.
- GRACE, J. B. 1993. The adaptive significance of clonal reproduction in angiosperms: an aquatic perspective. *Aquatic Botany*, 44: 159–180.
- HONNAY, O. & B. BOSSUYT. 2005. Prolonged clonal growth: escape route or route to extinction? *Oikos*, 108: 427–432.
- JANES, R. A., J. W. EATON & K. HARDWICK. 1996. The effects of floating mats of *Azolla filiculoides* Lam. and *Lemna minor* Kunth on the growth of submerged macrophytes. *Hydrobiologia*, 340: 23–26.
- KOLAR, C. S. & D. M. LODGE. 2001. Progress in invasion biology: predicting invaders. *Trends in Ecology and Evolution*, 16: 199–204.
- LANDOLT, L. 1999. Pleustonic communities with Lemnaceae in South America *Applied Vegetation Science*, 2: 7–16.
- LEMON, G. D., U. POSLUSZNY & B. C. HUSBAND. 2001. Potential and realized rates of vegetative reproduction in *Spirodela polyrhiza*, *Lemna minor* and *Wolffia borealis*. *Aquatic Botany*, 70: 79–87.
- RAI, V., N. V. SHARMA & A. K. RAI. 2006. Growth and cellular ion content of a salt sensitive symbiotic system *Azolla pinnata*-*Anabaena azollae* under NaCl stress. *Journal of Plant Physiology*, 163: 937–944.
- SCULTHORPE, D. 1967. *The biology of aquatic vascular plants*. Edward Arnold Ltd., London. UK.
- SHEPPARD, A. W., R. H. SHAW & R. SFORZA. 2006. Top 20 environmental weeds for classical biological control in Europe: a review of opportunities, regulations and other barriers to adoption. *Weed Research*, 46: 93–117.
- STATSOFT INC. 2001. *STATISTICA (data analysis software system), version 6*. Statsoft, <http://www.statsoft.com>.
- WAGNER, G. M. 1997. *Azolla*: a review of its biology and utilization. *The Botanical Review*, 63: 1–26.

- WETZEL, R. G. 2005. Invasive plants: the process within wetland ecosystems. In: *Invasive Plants: Ecological and Agricultural Aspects*. Inderjit (ed): 115–118. Birkhäuser, Germany.
- XIAO, K., D. YU & Z. WU. 2007. Differential effects of water depth and sediment type on clonal growth of the submersed macrophyte *Vallisneria natans*. *Hydrobiologia*, 589: 265–272.

