Annual Report 2018

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KNOWLEDGE FOR LIFE

Cover photo: summer student Aylin Kerim collecting *Bagous nodulosus* on *Butomus umbellatus* growing in CABI's artificial pond.

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Table of contents

Summary

1. Flowering rush (*Butomus umbellatus*) is a perennial aquatic plant of European origin that was introduced to North America as an ornamental over 100 years ago. It has developed into an aggressive invader of freshwater systems especially in the Midwestern and western states of the USA and western Canada. Since no effective control methods are currently available, a biological control project was initiated in spring 2013, and CABI in Switzerland subcontracted to conduct surveys for natural enemies in the area of origin of flowering rush. Currently, our work focuses on a weevil species in the genus *Bagous,* an agromyzid fly and one fungal pathogen. This report summarizes data collected by CABI in 2018.

2. We essentially completed sequential no-choice oviposition tests with *Bagous nodulosus*. Only one of the 45 test plant species offered so far was accepted for oviposition by female weevils, confirming the extremely narrow host range of the weevil. Because of the recent observation that larvae frequently leave the host and swim to other plants, we started larval establishment tests with 18 species. Although we are progressing well with these additional tests, preparation of a petition for release for *B. nodulosus* will be delayed by 1–2 years.

3. In 2018, we managed to establish a rearing colony of the agromyzid fly *Phytoliriomyza ornata.* We tested three different rearing set-ups and produced about 230 pupae from them. Together with additional field collected pupae, about 190 pupae are overwintering to be used for rearing, host-specificity tests and potentially an impact experiment in 2019.

4. Due to limited funding, work with the white smut *Doassansia niesslii* was reduced in 2018. Methodology of isolation and inoculation was further studied and the effect of media on *in vitro* growth and infectivity tested. Additional biotypes of flowering rush and additional test plants were tested for susceptibility to the first strain of *D. niesslii* we had collected in northern Germany. All common US genotypes of *B. umbellatus* were resistant to this strain. Another strain collected in eastern Germany will be tested in 2019, and we will intensify surveys for additional strains of the smut in Europe and Asia.

1. Introduction

Flowering rush (*Butomus umbellatus* L.) is a perennial aquatic plant that grows along lake shores and in slow-moving bodies of water, irrigation ditches and wetlands in temperate Europe and Asia. In several European countries, the plant is considered rare and endangered (Stöhr *et al.*, 2006; Raabe *et al.*, 2011). Fluctuating water levels favour the plant. It usually grows as an emergent with upright foliage in water up to 60–80 cm deep (Hroudová, 1989). In North America, where *B. umbellatus* was introduced more than 100 years ago as an ornamental, the common emergent form is found in up to 3-m-deep water and, in addition, submerged populations with flexible leaves suspended in the water column are known in up to 6-m-deep water (Jacobs *et al.*, 2011). Flowering rush is now considered an aggressive invader of freshwater systems, and is becoming an increasing problem in the Midwestern and western states of the USA and western Canada.

In irrigation ditches, flowering rush can increase ditch maintenance costs by reducing water flow. Plants reduce recreational opportunities along rivers and lake shores by interfering with boat propellers, swimming and fishing (Jacobs *et al.*, 2011). Flowering rush supports habitat for the great pond snail, which hosts parasites that cause swimmers' itch. Fish habitats are also affected where flowering rush forms dense stands in previously unvegetated or sparsely vegetated aquatic environments. This benefits introduced fish like largemouth bass, yellow perch and northern pike, which spawn in vegetated substrata, to the disadvantage of native cutthroat and bull trout, which require open water to spawn (Jacobs *et al.*, 2011). Stands of *B. umbellatus* may also threaten other shallow-water emergents such as the economically important *Zizania aquatica* (wild rice) (Brown and Eckert, 2005).

Two ploidy levels are known for *B. umbellatus*: diploids ($2n = 26$) and triploids ($2n = 39$) and both have been introduced in North America. Plants of the two ploidy levels differ in various ways. Diploids produce abundant fertile seeds, whereas triploids produce far fewer and sterile seeds (Krahulcová and Jarolímová, 1993). Despite heavy investment in seed production by diploids, little or no evidence of sexual recruitment was found in North America, suggesting predominantly clonal reproduction via bulbils (Fernando and Cass, 1997; Kliber and Eckert, 2005; Lui *et al.*, 2005), whereas North American triploids invest heavily in a large, carbohydrate-rich rhizome and appear to only propagate by rhizome fragmentation (Thompson and Eckert, 2004; Brown and Eckert, 2005).

In Europe triploids appear to be more common than diploids (Kliber and Eckert, 2005), and diploids are often found intermixed with triploids (Martin Hanzl, pers. comm.). In North America diploid populations are more frequent, especially in the Great Lakes region, while triploids appear to have a wider geographical distribution, which is probably due to their use in and escape from horticulture (Kliber and Eckert, 2005; Lui *et al.*, 2005). All populations analysed in the Midwestern and western USA so far have been triploid (Lui *et al.*, 2010).

Eckert *et al.* (2003) detected several different genotypes in North American populations with one dominant widespread genotype for each cytotype. In contrast, populations investigated by Poovey *et al.* (2012) all belonged to the same genotype. Further molecular analysis including North American and European populations is being carried out by Dr John Gaskin, US Department of Agriculture – Agricultural Research Service (USDA-ARS), Sidney, Montana. In preliminary AFLP analysis, he found eight

different genotypes in North America, and so far no match with a European population. The only match was for plants from a European nursery and a population from Illinois.

Several techniques are currently used for flowering rush control, such as mechanical control, planting desirable aquatic plants, managing water levels or chemical control, but all have to be repeated over several years, are costly, unsustainable and may involve high environmental risks (Jacobs *et al.*, 2011).

A biological control project against flowering rush was therefore started in spring 2013 on the initiative of Jennifer Andreas (Integrated Weed Control Project, Washington State University, USA), and CABI in Switzerland was subcontracted to conduct surveys for potential biocontrol agents. Because flowering rush is the only species in the family Butomaceae, the chances of finding very specific biocontrol agents are very high. We found six insect species in the literature recorded as monophagous on *B. umbellatus* and started working on four of them, two weevils and two flies. For one of the weevils, *Bagous validus,* we were not able to establish that *Butomus umbellatus* is its main field host, and one of the flies, *Hydrellia concolor,* does not appear to have much impact. Currently, we are therefore concentrating on the weevil *Bagous nodulosus* and the agromyzid fly *Phytoliriomyza ornata*. In 2016, we also started working on the white smut *Doassansia niesslii* in collaboration with plant pathologist Carol Ellison at our UK centre.

2. Work Programme for Period under Report

The work plan suggested for 2018 in the last annual report is outlined below. Certain objectives were adapted due to available funding, material and practicalities. Results are reported in subsequent sections.

***Bagous nodulosus* (Coleoptera, Curculionidae)**

- Continue to improve rearing success and collect more adults as necessary;
- Complete no-choice oviposition tests;
- Conduct single-choice tests with plant species that had moderate feeding under no-choice conditions;
- Repeat the impact experiment, starting earlier in the season.

***Bagous validus* (Coleoptera, Curculionidae)**

- Probably stop work with this species; only check and observe overwintered plants exposed to weevils at CABI.

***Phytoliriomyza ornata* (Diptera, Agromyzidae)**

- Establish a colony at CABI;
- Study its biology and develop methods for host-specificity tests;
- Provided enough flies are available, carry out an impact experiment.

***Doassansia niesslii* (Basidiomycota)**

- Obtain plant material of all main North American genotypes for testing with isolates of *D. niesslii* (particularly genotype 4);

- Test new isolate of *D. niesslii* from Elsnig, Saxony, Germany;
- Collect samples of *D. niesslii* from other sites (countries) in Europe where the pathogen has been recorded;
- Investigate culturing techniques to retain sporidia infectivity;
- Confirm underwater infectivity of sporidia;
- Continue host-specificity tests.

3. *Bagous nodulosus* GYLLENHAL (Coleoptera, Curculionidae)

Since the beginning of the project, we considered the semi-aquatic weevil *Bagous nodulosus* as the most promising candidate for biological control of flowering rush, because a single larva causes the most impressive damage in leaves and rhizomes of *Butomus umbellatus*. The weevil is univoltine and development from egg to adult takes about two months. Since we found developing larvae in field collected samples from May to September, the oviposition period must last from April to July (Häfliger *et al*., 2018). An impact experiment carried out in 2017 led us to realize that larvae commonly leave the host plant and swim to infest surrounding flowering rush plants. The adult weevils spend most of their time underwater, can live up to at least two years, and overwinter on plant debris underwater.

Plate 1. Adult *Bagous nodulosus* underwater (left; photo: Tim Haye) and swimming first instar larva (right).

3.1 Host-specificity tests

3.1.1 Sequential no-choice oviposition tests

METHODS As in previous years, we continued sequential no-choice oviposition tests using cut leaves. Adults were collected in May in Germany, Serbia and Slovakia, and we tried to set up at least two valid replicates of each population for each test plant species between May and July. To ensure that only egg-laying females were used for this test, females were kept individually for two days in plastic cups (diameter 5.5–6.5 cm, height 8 cm) with 1–2 cm of water and two cut leaves of flowering rush. Only females that laid at least one egg within two days were used. Cut leaves of test plants were individually exposed to ovipositing females for two days in 1.3-litre plastic cylinders half-filled with water. Females were then placed back onto cut leaves of

flowering rush to verify that they were still laying eggs. Tests were only considered valid if the female laid at least one egg on the control (flowering rush) within two days of the test. Females that were still laying eggs were subsequently exposed to another set of test plants. Eggs found during the tests were used to supplement our rearing colony.

RESULTS Using this method, 90 additional replicates were set up in 2018, including three new species. Between 2014 and 2018, a total of 45 test plant species, 29 native to North America, were exposed to *Bagous nodulosus* females (Table 1). On average, females laid exactly the same number of eggs on North American (genotype 1) and European *Butomus umbellatus* (all triploid). No eggs were found on the 45 test plant species, except in one case on *Baldellia ranunculoides*, a European species.

3.1.2 No-choice larval establishment tests

Since we now know that larvae commonly leave the host plant to swim to neighbouring *Butomus* plants, it is now necessary to also conduct tests with larvae. Preliminary tests showed that larvae readily start feeding in cut *Butomus* leaf pieces, when these are offered in a cylinder filled with water. We also commonly found larvae swimming and crawling in plastic cups about five to ten days after eggs were laid on cut leaves of flowering rush. Based on these observations, we developed the following method to test early larval establishment and feeding.

METHODS Leaf pieces of *B. umbellatus* were exposed in 1.8–dl plastic cups to ovipositing females for 2–5 days. After about five days, cups were checked twice a day for swimming larvae, and pairs of larvae were transferred into 1.3-litre cylinders half filled with water containing 1–2 cut leaves or stem pieces (6- to 20-cm long) of a test plant. On each day a series of test species was set up, a control with two leaves of *Butomus* was established in parallel. After five days, plant material, water and cylinders were checked for dead and live larvae and plants for feeding marks.

Plate 2. Set-up of oviposition and larval establishment tests with *Bagous nodulosus.*

RESULTS Using this method, we exposed 18 test plant species in 1–11 replicates (Table 1). On seven species we found live first instar larvae five days after set-up, and on five species we also found dead or live second instar larvae. However, in most cases larvae were found floating in the water or resting on the surface of the plant, and only two species, *Limnobium laevigatum* and *Hydrocharis morsus-ranae* (both Hydrocharitaceae not native to North America), showed obvious feeding marks or limited mining (Table 1, last column).

Table 1. Results of sequential no-choice oviposition tests with *Bagous nodulosus* carried out between 2014 and 2018 and no-choice larval establishment tests started in 2018. For each test plant species and test we aim for six replicates. Numbers in red indicate that additional replicates are necessary.

Plant species[a]	Oviposition				Larval establishment		
	No. replicates set up	No. valid replicates	No. eggs per replicate	Adult feeding[b]	No. replicates set up	No. live larvae	Larval feeding[b]
Order Alismatales							
Family Butomaceae							
Butomus umbellatus (European)	3154		1.4	+++	33	38	+++
Butomus umbellatus (American)	113		1.4	+++	1	2	+++
Family Alismataceae							
Alisma plantago-aquatica	13	7	0	+			
Alisma subcordatum[a]	10	6	0	-	5	0	-
Alisma triviale[a]	20	6	0	+	7	5	-
Baldellia ranunculoides	22	12	0.8	+	6	1	+
Damasonium californicum[a]	13	5	0				
Echinodorus berteroi[a]	25	13	0	+	8	3	+
Echinodorus cordifolius[a]	31	11	0	+	5	0	-
Sagittaria cuneata[a]	14	7	0	+	4	0	-
Sagittaria graminea[a]	19	6	0	+	6	0	-
Sagittaria latifolia[a]	29	10	0	(+)	4	0	-
Sagittaria platyphylla[a]	11	9	0	+			
Sagittaria rigida[a]	13	7	0	-	5	0	-
Order Hydrocharitales							
Family Hydrocharitaceae							
Blyxa aubertii	3	2	0	++			
Elodea bifoliata[a]	6	6	0	-			
Elodea canadensis[a]	20	8	0	+			
Elodea densa	17	7	0	+	3	0	-
Elodea nutallii[a]	11	8	0	-			
Hydrilla verticillata	19	10	0	+			
Hydrocharis morsus-ranae	14	6	0	+	10	3	++
Limnobium spongia[a]	11	6	0	+			
Limnobium laevigatum	11	4	0	++	11	6	++

Plant species[a]	Oviposition				Larval establishment		
	No. replicates set up	No. valid replicates	No. eggs per replicate	Adult feeding[b]	No. replicates set up	No. live larvae	Larval feeding[b]
Najas guadalupensis	7	4	0	-			
Vallisneria americana[a]	15	10	0	++	5	0	-
Order Nymphaeales							
Family Ceratophyllaceae							
Ceratophyllum demersum[a]	23	6	0	+			
Family Nymphaceae							
Nuphar lutea[a]	17	7	0	-			
Nuphar advena[a]	5	3	0	-			
Nymphaea odorata	12	6	0	-			
Order Haloragales							
Family Haloragaceae							
Myriophyllum spicatum	16	9	0	+			
Order Najadales							
Family Potamogenaceae							
Potamogeton amplifolius[a]	13	3	0	+	5	1	-
Potamogeton natans	12	6	0	+	8	1	-
Potamogeton lucens	12	6	0	+			
Potamogeton richardsonii[a]	12	8	0	-			
Stuckenia pectinata[a]	12	9	0	-			
Order Liliales							
Family Pontederiaceae							
Heteranthera dubia[a]	11	6	0	-			
Family Iridaceae							
Iris pseudacorus	19	10	0	-			
Iris virginica[a]	9	5	0	-	5	0	-
Order Cyperales							
Family Cyperaceae							
Carex obnupta[a]	16	6	0	-			
Schoenoplectus acutus[a]	18	9	0	-			
Schoenoplectus tabernaemontani[a]	29	6	0	+	5	0	-
Family Poaceae							
Glyceria borealis[a]	26	11	0	(+)			

Plant species[a]	Oviposition				Larval establishment		
	No. replicates set up	No. valid replicates	No. eggs per replicate	Adult feeding[b]	No. replicates set up	No. live larvae	Larval feeding[b]
Oryza sativa	12	7	0	-			
Phalaris arundinacea	17	7	0	-	4	0	-
Zizania aquatica[a]	14	8	0	-			
Order Myrtales							
Family Lythraceae							
Lythrum salicaria	8	6	0	-			
Order Polygonales							
Family Polygonaceae							
Polygonum amphibium[a]	11	7	0	+			

[a] Plant species native to North America.
[b] - = no feeding, + = minor feeding on single leaves, ++ = some feeding on several replicates, +++ = major feeding on most replicates, brackets = not sure.

3.1.3 Discussion and conclusions

Sequential no-choice oviposition tests were nearly completed in 2018. Only a few replicates for seven species are missing, 14 replicates in all. Since it appears to be difficult to obtain *Blyxa aubertii*, we will order *B. japonica* and complete the tests with this species.

The results confirm the extremely narrow host range of *Bagous nodulosus*. Apart from seven eggs laid on *Baldellia ranunculoides* in one out of 12 replicates, no eggs were laid on any other test plant species. We consider the eggs found on *B. ranunculoides* to be an artefact caused by test conditions. In addition, this species is considered as introduced in North America, and its thin petioles, leaves and stems would not allow larval development of the weevil.

Adult feeding observed on test species is in general not much more than probing. Three species in the family Hydrocharitaceae showed some additional feeding marks, but not enough to cause significant damage. Single-choice and larval establishment tests will give further indications as to whether *Bagous nodulosus* would damage these species under more natural conditions or not.

The method developed for no-choice larval establishment tests works well. Considering the short test period, coupled with the fact that *B. nodulosus* larvae appear to be able to survive for several days without feeding, it was not too surprising to find live first instar larvae on some of the test species. We are therefore planning to extend the exposure time next year to seven days and design larval development tests on potted plants with species supporting development to second instars. We also have to keep in mind that larvae were feeding for a few days on *Butomus umbellatus* before being exposed to the test plants.

Two species in the family Hydrocharitaceae that form floating leaves (*Limnobium laevigatum* and *Hydrocharis morsus-ranae*) showed limited mining and will need to be included in longer development tests in 2019. We used the non-native *L. laevigatum* instead of the native North American *L. spongia* for our tests in 2018, because this species was easier to obtain. Since *L. laevigatum* seems to at least partly support some larval development, it will be important to include the native *Limnobium* species in further tests. Therefore, a shipment of *L. spongia* from Nate Harms (US Army Engineer Research and Development Center, Vicksburg, MS) is scheduled for spring 2019.

3.2 Impact experiment

Because we do not fully understand the natural behaviour of *Bagous nodulosus* larvae, we have not yet been able to properly evaluate impact of larval feeding on flowering rush. An impact experiment carried out in 2017 showed a biomass reduction of 33% mainly due to adult feeding. In spring 2018, we set up another impact experiment, drawing on our new knowledge about the biology of *B. nodulosus*, with the aim to measure impact of adult and larval feeding.

METHODS At the end of April 2018 (six weeks earlier than in 2017), we set up an experiment in a pool (2.1 m by 4 m by 0.8 m) filled to 50 cm with water. We set up 34 *Butomus* plants (genotype 1 from South Dakota) in 3-litre pots and recorded number of emerged sprouts, number of leaves and length of the longest leaf and made sure that means of these measurements did not significantly differ between treatments at the time of experimental set-up. All 34 experimental plants were covered with gauze bags (1 mm mesh width) and three pairs of *Bagous nodulosus* each were released on

half of them. To avoid contamination of control plants (onto which no weevils were released) by swimming larvae, we separated controls and exposed plants with a dense gauze. On both sides of the gauze, we grouped plants in six rows separated by uncovered “trap” plants (36 each side) to give swimming larvae the opportunity to find fresh plants to infest (Plates 3 and 4).

Plate 3. Set-up of impact experiment with *Bagous nodulosus* after removal of gauze bags (left: controls with flowering plants, right: exposed plants, no flowers).

To facilitate larval dispersal, gauze bags were removed four weeks after set-up and weevils collected from exposed plants (only nine weevils out of 107 were not found). Number of leaves was recorded again for all experimental and some trap plants.

After eight weeks, all 34 experimental plants and 57 trap plants (91 in total) were dissected and any larvae, pupae, adults or mines were recorded. Since 12 trap plants were from a European population that grew taller than the South Dakota population, these were excluded from the analysis. Before dissection, number of leaves, number of sprouts and length of leaves were recorded for each pot. Plant material was dried at 80°C for 48 hours and dry weight recorded separately for above- and below-ground biomass. Since this work extended over five weeks, we took care to analyse plants from all treatments within the same time period. Means were compared with a one-way ANOVA using SPSS 25.0.

RESULTS After four weeks of exposure to weevils, the number of leaves did not significantly differ between plants exposed to adults and control plants (Table 2), but five plants were flowering in the control group and none in the group exposed to weevils. After 2–3 months, plants exposed to weevils had 46% more leaves and 34% higher above-ground biomass, but 48% less below-ground biomass, than control plants. Larval feeding on trap plants also resulted in 32% more leaves than on plants without larvae. Trap plants with larvae had higher above-ground biomass and lower below-ground biomass than plants without larvae, but differences were not significant

(Table 2). Plants exposed to weevils and trap plants intermixed with them both supported on average the development of one weevil (maximum five weevils per plant). The gauze separating control and exposed plants worked fairly well, and only few experimental control or trap plants were attacked, mostly by only one weevil larva or adult.

Table 2. Results of impact experiment with *Bagous nodulosus.*

Treatments	No. leaves after 4 weeks	No. leaves after 8-13 weeks	Above-ground biomass	Below-ground biomass	No. larvae (or pupae/adults) developed
Control	15.7 ± 1.9	21.2 ± 1.8	5.3 ± 0.7	15.1 ± 1.0	0.1 ± 0.1
Exposed	12.8 ± 1.2	31.1 ± 2.5	7.1 ± 0.6	7.9 ± 0.6	1.0 ± 0.3
Statistics	$P = 0.188$	$P = 0.003$	$P = 0.079$	$P < 0.001$	$P = 0.007$
"Trap" control	22.4 ± 2.3	24.7 ± 1.9	5.5 ± 0.6	12.1 ± 1.2	0.1 ± 0.1
"Trap" exposed	22.4 ± 1.4	32.7 ± 2.0	6.4 ± 0.3	11.4 ± 1.1	1.2 ± 0.3
Statistics	$P = 0.983$	$P = 0.007$	$P = 0.137$	$P = 0.682$	$P = 0.002$

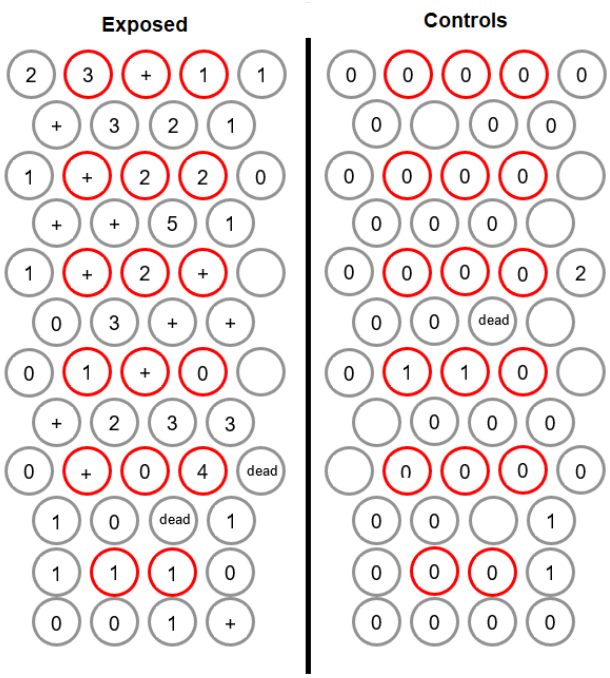

Plate 4. Experimental set-up and number of weevils that developed in impact experiment (red circles = plants covered with gauze bags; grey circles = trap plants not covered with gauze; empty = not analysed; 0 = no mines; + = only early instar mines; 1–5 = number of adults, pupae or third instar larvae; black line = separation of infested and control plants by fine-mesh gauze).

DISCUSSION Results of the impact experiment carried out in 2018 differed from the one in 2017. Most of the differences can be explained by changes in the set-up. In 2017, plants were exposed for two weeks longer (six instead of four weeks) to more pairs of *B. nodulosus* (five instead of three), and plants were analysed immediately after gauze bags were removed. Therefore, we recorded a 30% reduction of above-ground biomass in 2017, while this was compensated for by regrowth of nearly 50%

additional leaves one to two months after removing the weevils in 2018. Very surprising was the nearly 50% reduction of below-ground biomass found in plants exposed to adults in 2018 (none was observed in 2017). Since only rhizomes were used for analysis in 2017 but all below-ground parts in 2018, the difference could reflect lower fine root production by exposed plants, which we would have missed in 2017. While it appears logical to explain this difference by re-allocation of below-ground resources to compensate for above-ground biomass loss due to adult feeding, the magnitude of the difference in below-ground biomass seems excessive. We are not aware of any adult feeding of *B. nodulosus* on fine roots. However, we cannot fully exclude that we could have missed it.

It was disappointing to see that again no impact of larval feeding could be shown. Nevertheless, more information about larval behaviour of *B. nodulosus* was obtained that can be used to develop better experimental designs. We commonly found exit and entrance holes of first instar larvae in the leaves. The mines were often short, but sometimes long, extending from an entrance hole near the leaf tip to the base of the leaf. Larvae apparently feed as 2nd instars down to the rhizome and move about 10-20 cm up as 3rd instars in the next leaf where they form a pupal chamber.

Adding trap plants that were not exposed to adults to the experimental set-up should have allowed us to separate impact due to adult feeding from impact due to larval feeding. With on average one adult developing per plant, the density appears to be too low to result in impact. There are still many questions to answer: why do larvae frequently take the risk and leave a leaf only a few days after egg hatch, although there would be sufficient resources to support full development of even several weevils in one plant? Could this be triggered by plant defences forcing larvae to leave? If yes, could this defence also be responsible for the fact that we never reach high larval densities in our experiments and for the low development rates in our rearing colony? We plan to develop experiments to answer these questions in 2019.

3.3 Rearing

Overwintering of adult weevils on submerged potted plants covered with gauze bags works very well. Winter survival of up to 80% was observed (Häfliger *et al.*, 2016). However, retrieving adults from overwintering plants can be quite time consuming. Although females readily lay eggs on any *Butomus umbellatus* plant offered (independent of genotype or ploidy level), and although full larval development to adulthood was possible under rearing conditions, success rates remain rather low (< 5%). We first assumed that unsuitable water quality, water temperatures or plant quality was responsible for high loss of larvae during rearing. Our new observation that larvae commonly take the risk of switching plants, although they would have plenty to feed on if they did not, suggests plant defences might be implicated. We plan to test this hypothesis in 2019.

We found the so-far most efficient rearing technique by accident. When we set up ovipositing weevils on plants covered by gauze bags in an artificial pound, other plants kept in the pond became infested by swimming larvae. Thus, in 2018 we were able to collect about 200 weevils reared this way. Assuming many of these weevils had been able to oviposit before being collected, we expect to collect a similar number of weevils from this pond in 2019. Nevertheless, we will continue to search for rearing techniques that need less space and could also be used in quarantine.

At least 80 adults are overwintering on plants covered with gauze bags in a pool.

4. *Bagous validus* ROSENHAUER (Coleoptera, Curculionidae)

As mentioned in the last annual report, we decided to stop working with this species closely related to *Bagous nodulosus*, because we were not able to confirm larval development on flowering rush (Häfliger *et al*., 2018). We nevertheless continued keeping adults on flowering rush plants covered with gauze bags and found individuals surviving after two years under these conditions. Given the results of the impact experiment with *B. nodulosus* in 2018 (see section 3.2), it could be worthwhile to investigate the potential of *B. validus* larvae feeding externally on fine roots of *Butomus umbellatus*, which we might have overlooked in the past.

5. *Phytoliriomyza ornata* (MEIGEN) (Diptera, Agromyzidae)

We found larvae and pupae of the agromyzid fly *Phytoliriomyza ornata* during dissections of plants at many of our European field sites, but also in samples from Kazakhstan collected by our colleague Sonja Stutz in May 2018. At the beginning of the project, we expected *P. ornata* to have a lower impact on growth of *B. umbellatus* than the weevil *Bagous nodulosus*, because the mines were less conspicuous. However, much higher larval densities were found for *P. ornata* than for *B. nodulosus*, and we saw plants wilting three weeks after exposure to a single female for three days (Häfliger *et al*., 2018).

Plate 5. *Phytoliriomyza ornata* ovipositing on a *Butomus umbellatus* leaf (left) and set-up on plants covered with gauze bags for rearing (right).

The fly seems to have one to two generations per year, developing at the same time both transparent pupae, from which adults emerge the same year and black overwintering pupae. The factors determining the type of pupa are still unknown.

METHODS In fall 2017, about 120 pupae dissected from leaves collected in Germany and about 60 pupae obtained from our rearing colony were set up for overwintering in Petri-dishes stored in a styrofoam box in a wooden shelter at ambient temperatures.

We used three different set-ups for rearing: (1) as successfully tested in 2017, single pairs were set up on potted plants between 23 April and 9 May 2018 (North American populations, genotype 1 from Montana) covered with a plastic cylinder (diameter 10 cm, height 26 or 37 cm) (*n* = 21) (Table 3: “cylinders”). Cotton pads soaked in either water, honey water or honey and pollen were provided as a food source. After 3–4 days flies (if still alive) were moved to a new plant with the same set-up (*n* = 11). In addition, we tested two more natural set-ups with pots placed either (2) in a 10-litre bucket filled with water (Table 3: “gauze bags submerged) or (3) in a saucer and just watered from the bottom by regularly refilling (Table 3: “Gauze bags"). Flies in these set-ups were only in six cases moved to a new plant after one week. A total of 52 plants were set up using one of the three methods and protected from rain by placing in a polytunnel in our garden. After 4–6 weeks, plants were dissected for pupae. Because dissection for pupae is very time consuming, we tested two alternative methods to extract pupae from leaves: (1) ten plants were set up in photoeclectors (whole plant covered by a black plastic bag with a small opening covered by a plastic cup), hoping that emerging flies would be attracted by light and that we could simply collect them in the plastic cups; (2) allowing cut leaves containing pupae to decay in water for two months, hoping to retrieve potentially floating pupae.

During field trips in September 2018 in northern Germany and Hungary, additional flowering rush plants were collected and dissected for overwintering pupae of *P. ornata*, in order to have sufficient flies available in 2019.

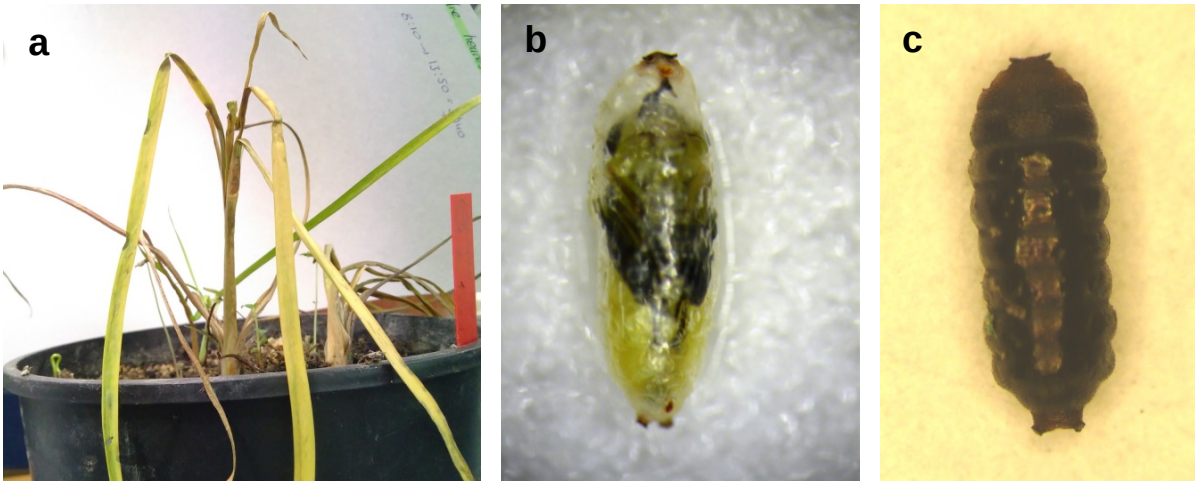

Plate 6. Plant damaged by larval feeding of *Phytoliriomyza ornata* (a), transparent pupa; adult emerges the same year (b) and black overwintering pupa; adult emerges the following year, (c).

RESULTS Between 21 April and 9 May 2018, 95 flies and 46 parasitoids emerged from 189 overwintering pupae. From 45 pairs of *P. ornata* set up on plants, about 210 pupae were found during dissections with a maximum of 20 pupae per plant. Another 24 pupae were obtained from seven pairs of the second generation emerging between mid-June and early July. Flies set up on gauze covered flowering rush plants placed in water buckets (see Plate 5) resulted in highest numbers of pupae (Table 3). Providing additional food in the form of honey or pollen did not lead to significantly higher numbers of pupae (water 3.7 ± 1.0; honey 5.4 ± 0.9; honey and pollen 3.0 ± 0.9; *P* = 0.209). We found a positive correlation between the number of pupae per plant and the number of leaves per plant (Pearson correlation *r* = 0.339, *n* = 52, *P* = 0.016).

Table 3. Rearing success of *Phytoliriomyza ornata* with three different set-ups: plants covered by cylinders or gauze bags placed on saucers and submerged plants covered by gauze bags).

Set-up	Average no. pupae per plant	Transparent or brown pupae (% total pupae)	Emerged 2nd generation (% transp./brown pupae)
Cylinders (*n* = 13)	2.1 ± 0.5	52%	46%
Gauze bags (*n* = 24)	5.0 ± 0.7	41%	30%
Gauze bags submerged (*n* = 4)	10.3 ± 3.0	5%	75%

In addition to transparent and black pupae (Plates 6b and 6c), we also found brown to nearly black pupae from which adults also emerged after a few weeks. The number of overwintering pupae was higher than the number of pupae from which adults emerged this year. We also found transparent pupae on plants exposed to second generation flies, but no adults emerged from them in 2018.

Unfortunately, alternatives tested to extract pupae from leaves were not very effective. No flies emerged from plants set up with photoeclectors and only a few pupae were found floating on the water surface after letting leaves decay for two months.

DISCUSSION The highest rearing success was achieved on gauze-covered, submerged plants. We assume that this was due to either better plant quality or a higher water availability under these conditions. But it could also be an indirect effect of better performance on plants growing under better conditions. The fact that we found significantly fewer pupae on field collected plants in 2018 compared to 2017 would support this. Many usually submerged field sites were mostly dry in fall 2018 and flowering rush plants were visibly stressed and less common than in other years.

Up to 20 pupae were found in one plant. Thus, *P. ornata* can reach much higher densities on *Butomus umbellatus* than the weevil *Bagous nodulosus*.

Host-specificity tests will most probably need to be set up as development tests on potted plants, since it is extremely difficult to detect eggs even on controls.

6. *Doassansia niesslii* DE TONI (Basidiomycota)

The white smut *Doassansia niesslii* is a leaf pathogen of *Butomus umbellatus* and was identified as a potential biocontrol agent for this invasive plant in 2015 (Häfliger *et al.*, 2016). The white smut has only been recorded to infect flowering rush in the literature, and there are records from the Czech Republic, Czechoslovakia, Poland, Russia and Sweden, as well as Germany (Farr and Rossman, undated). White smuts are hemi-biotrophic fungal pathogens; their life cycle can only be completed on the host plant, but there is a necrotrophic phase of the life cycle that can grow in culture (on agar). White smuts generally prefer wet environments and overwinter as resting spores in the plant tissue; they can be very damaging. An example of the successful control of an invasive plant with a white smut is mistflower (*Ageratina riparia*) in Hawaii and New Zealand (Fröhlich *et al.*, 2000).

Doassansia niesslii has two spore states: the sexual or teleomorphic state and the asexual or anamorphic state. The sexual state is a resting spore, forming completely within the leaf tissue (mesophyll) and once the leaf tissue has senesced enters a period of dormancy (over winter) before germination can occur. The resting spores are only liberated by rupture of old and decaying litter. Under laboratory conditions, the teliospores were able to infect leaves growing underwater. This indicates that the white smut should be able to infect completely submerged *B. umbellatus* populations. In addition, we observed plant die-back four weeks after infection, indicating potential strong impact of the pathogen. The asexual state forms as pycnidia just under the epidermis and releases spores outside the plant through leaf stomata. These spores germinate immediately and infect new leaf material, causing severe damage throughout the growing season. It is unknown yet if these spores are able to infect plants growing completely submerged, but work is underway to investigate this.

Plate 7. Spore balls (teleomorph stage) of white smut (left); leaf die-back following infection underwater (right).

6.1 General isolation and inoculation methodology

Good, reliable infection of susceptible plants with the white smut can be obtained on agar culture using freshly produced sporidia isolated from the asexual state. Unfortunately, the infectivity of the sporidia was found to decrease over time; viability was maintained for up to eight weeks for sporidia growing on potato–carrot agar (PCA) at 19°C. However, sporidia taken from the edge of 4- to 8-week-old colonies and subcultured onto fresh agar lost the ability to infect *B. umbellatus*. This may be due to the virulence gene being turned off after prolonged subdivision of cells *in vitro*. Therefore, recently isolated sporidia need to be produced for each plant inoculation.

Sections of leaves (approximately 4 cm long) showing significant *D. niesslii* symptoms of infection, but prior to leaf senescence, were cut from plants inoculated 6–10 weeks previously. Sections were surface sterilized by wiping with a piece of tissue soaked in ethanol, or submerging in sodium hypochlorite for 3 minutes, then washing in sterile distilled water (SDW). With the aid of a dissecting microscope, a slit was made along the leaf, piercing to about half the thickness of the tissue, using a sterilized scalpel blade, in an area where pycnidia could be seen just under the epidermis. The pycnidia were exposed by pealing back the upper leaf surface and they were then extracted using a hypodermic needle. The pycnidia were placed on PCA containing antibiotics and kept at 19°C. At least six agar plates, each with approximately six pycnidia placed on them, were prepared for each plant inoculation. This is because not all pycnidia produced sporidia, and there were inevitably some contaminated plates. After a week,

small (1-mm diameter), white, slimy sporidial colonies could be seen developing from about 25% of the pycnidia.

After four weeks, the colonies (approximately 5 mm in diameter) were picked off and dispersed in 0.1% agar ('sloppy agar') containing 0.05% v/v Tween 80 (a surfactant to help sporidia disperse). The agar helped inoculum adhere to the vertical leaves of *B. umbellatus*, rather than run off on to the soil. The sporidial suspension was brush inoculated (using a camel hair paint brush) onto the leaves of plants and placed in a dew chamber set at 15°C for 24 hours to allow for infection. Plants were then maintained in a greenhouse chamber with supplemented lighting and a minimum night temperature of 17°C and maximum day temperature of 25°C.

6.2 Effect of media on *in vitro* growth and infectivity of *D. niesslii* (ex Bremen Germany)

METHODS Sporidial growth on PCA is very slow, and colonies remain small; presumably nutrients are exhausted within the colony area. However, colonies growing from subcultured sporidia lose their viability. Also, it is known that the viability of *in vitro* produced spores from plant pathogens (particularly hemi-biotrophic species) is dependent on the nutrient status on the agar media; nutrient rich media are known to yield spores with low viability (Yu *et al*., 1998). An experiment was set up to investigate the effect of media type on growth and viability of sporidia. Freshly harvested spores were grown on four types of media with different nutrients (Table 4) and the resultant sporidia inoculated (as described in section 6.1) onto *B. umbellatus* plants; 3–6 plants were inoculated per media.

Table 4. Sporidial growth on agar media with different nutrient levels and ability of resulting sporidia to infect *Butomus umbellatus* (ex Bouchie Lake, Canada; genotype 2)

Agar media type	Relative speed of colony development (weeks) to maximum size (mm)	Colony growth type on media	Level of susceptibility of *Butomus umbellatus*
PCA (potato–carrot agar)	Slow (4 weeks, 5 mm)	Slimy appearance with brain-like growth form. Mycelium sometimes develops	High (many coalescing lesions, with numerous spore balls in leaf tissue)
SDA (Sabouraud dextrose agar) made with distilled water	Medium (3 weeks, 10 mm)	Slimy appearance with brain-like growth form	High
'V8' (vegetable juice agar)	Medium (3 weeks, 5 mm)	Slimy appearance with brain-like growth form	Medium (a few lesions with many spore balls)
TSA (tryptic soy agar) – highly nutritious	Fast (2 weeks, 10 mm)	Slimy appearance with brain-like growth form	Low (one lesion, few spore balls)

RESULTS The experiment confirmed that rich media may reduce the viability of the sporidia even though more sporidia are produced (Table 4).

6.3 Update on *B. umbellatus* biotype susceptibility to *D. niesslii* ex Bremen Germany

METHODS Plants were inoculated with sporidia as described in section 6.1 above. At least four plants of each genotype were inoculated with the sporidial suspension, and this was (or will be) repeated for each genotype at least once. Additional plants of each population were brushed with agar carrier only, and placed in a separate dew chamber for 24 hours as controls.

Table 5. Updated results of inoculations of *Butomus umbellatus* with *Doassansia niesslii* (blue = 2018 inoculations).

Population	Probable ploidy level	North American AFLP genotype	Number of times tested (4–6 plants each time)	Susceptibility to *D. niesslii*
Bremen, Germany (site 1)	---		6	Moderately susceptible
Bremen, Germany (site 2)	---		3	Susceptible
Bremen, Germany (site 4)	---		4	Strongly susceptible
Water Garden Plants, UK supplier	---		3	Immune
Slovakia	Triploid		4	Immune
Vojany, Slovakia	Diploid		4	Immune
Horticultural supplier, Switzerland	---		1	Immune
Bouchie Lake, Canada (used as control in all experiments)	Triploid	2	Numerous (10+)	Strongly susceptible
Montana, USA	Triploid	1	9	Resistant (a few spore balls in dead outer [older] leaves)
South Dakota, USA	Triploid	1	5	Immune
Wisconsin, USA	Triploid	1	2	Immune
Staines, Surrey, UK			3	Strongly susceptible
Elsnig (near Leipzig), Saxony, Germany	---		3	Weakly susceptible (low infection level & few spore balls produced)
Georgia 1, 2 and 4	---		1	Resistant
New York state, USA	Diploid	4	1	Resistant [a]
Maine, USA	Diploid	4	1	Resistant [a]
Ohio, USA	Diploid	4	1	Resistant [a]
Minnesota, USA	Diploid	5	1	Resistant [a]

[a] Not conclusive, plants too young, need to repeat

RESULTS Additional inoculations in 2018 showed that genoypes 4 and 5 of *B. umbellatus* are also resistant to the *D. niesslii* strain from Bremen, Germany (Table 5). None of the controls (inoculated with agar only) showed symptoms, confirming the validity of the method.

6.4 Host-specificity testing

METHODS The North American native species *Alisma subcordatum*, *Echinodorus cordifolius* and *Sagittaria rigida* were sent to CABI-UK from CABI-CH and were screened for their susceptibility to the white smut isolate from Bremen, Germany. Plants were inoculated with sporidia as described in section 6.1 above. Multiple plants (2–6) were inoculated with the sporidial suspension for each test plant species; this has been (or will be) repeated for each species. A susceptible biotype of *B. umbellatus* (Bouchie Lake, Canada) was included as a control, to prove that the spores were infective.

RESULTS The three new plant species tested in 2018 were immune to infection by the pathogen (no symptoms observed) (Table 6), This still requires to be repeated for *A. subcordatum* and *S. rigida,* since only one round of tests have been conducted to date. The controls were fully susceptible.

Table 6. Updated results of host-specificity testing of *Doassansia niesslii* (blue = 2018 inoculations).

Plant species	Number of times tested (2–6 plants per screening	Reaction to *D. niesslii*
Alisma plantago-aquatica	5	Immune
Sagittaria graminea[a]	3	Immune
Carex obnupta[a]	3	Immune
Alisma subcordatum[a]	1	Immune
Echinodorus cordifolius[a]	3	Immune
Sagittaria rigida[a]	1	Immune
Butomus umbellatus	10+	Fully susceptible

[a] Plant species native to North America.

6.5 Discussion and conclusions

Doassansia niesslii is a damaging potential biocontrol agent for *B. umbellatus* in North America. So far, a single strain of this white smut, from Bremen, northern Germany, has been studied. Work has continued on *in vitro* culturing of sporidia produced by the asexual stage of this pathogen. A preliminary study on the effect of different agar culture media on sporidial production and their ability to infect *B. umbellatus* (viability) was undertaken. The results indicated that a high number of sporidia are produced on nutrient rich agar, but that the viability of these spores is reduced. Spore production in liquid culture will be investigated in 2019–2020.

Critical to this research is the need to collect additional strains of the white smut, and test their infectivity on the different genotypes of flowering rush invading North America. The strain from Bremen is fully compatible with North American AFLP genotype 2

(currently restricted to Bouchie Lake, Canada). However, it does not infect genotypes 1 (the most common genotype), 4 or 5. A new isolate of *D. niesslii* was collected in 2017 near Elsnig, Saxony, Germany, and will be tested in 2019.

No matches, using ALFP analysis, have been found between the North American genotypes and European samples from Germany, Czech Republic, Slovakia, Hungary, eastern Poland, Serbia, Georgia and Switzerland. However, the earliest record of flowering rush in North America was in 1897 in Châteauguay, near Montreal, Quebec. As this area was where many French immigrants originally settled, it is possible that flowering rush was introduced from France, as an ornamental or in ballast (Cao *et al.*, 2018). Most of the French immigrants to this region travelled on ships leaving from Le Havre in the 1800s. Since no plant samples from France have been included in the molecular study, as yet, it is proposed that the first surveys in 2019 will focus on the Seine tributaries and wetlands of the Parc Naturel Régional des Boucles de la Seine Normande, in northern France. Although there are many records of *B. umbellatus* from this region, the white smut has not been recorded. Since the smut has also been recorded from Sweden, and plants from this area have not been included in the AFLP analysis, an additional survey is planned to be undertaken in this area in 2019. Our project partner Nate Harms (US Army Engineer Research and Development Center, Vicksburg) is planning to collect leaf samples in China and also search for the white smut there.

Host-specificity testing also continued in 2018; three additional native North American species were screened for their susceptibility to *D. niesslii*. They were found to be immune, but need to be retested to confirm this, following standard testing procedures. It is highly unlikely that this pathogen will infect any other plant species. *Doassansia niesslii* is only recorded from *B. umbellatus,* and *B. umbellatus* belongs to a single-species genus.

7. Work Programme Proposed for 2019

Bagous nodulosus (Coleoptera, Curculionidae)

- Continue to improve rearing success and collect more adults as necessary;
- Complete no-choice oviposition tests;
- Conduct single-choice tests for plant species with moderate feeding;
- Continue no-choice larval establishment tests;
- Conduct full development tests using potted plants of species that supported development to second instar;
- Set up new impact experiment, taking into account lessons learned from previous experiments.

Phytoliriomyza ornata (Diptera, Agromyzidae)

- Maintain and increase rearing colony at CABI;
- Start host-specificity tests;
- Provided a sufficient number of flies is available, conduct experiment to quantify impact.

***Doassansia niesslii* (Basidiomycota) (pending additional funding)**

- Undertake surveys for additional strains of *D. niesslii*, especially in France and Sweden;
- Test new isolate of *D. niesslii* from Elsnig, Saxony, Germany;
- Test isolates collected in 2019 for susceptibility of main North American invasive genotypes;
- Continue investigation of underwater infectivity of *D. niesslii* spores;
- Continue investigation into sporidial *in vitro* mass production;
- Continue host-specificity testing.

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