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Evaluation of a *Puccinia* Rust as a Potential Biological Control Agent of *Fallopia japonica*

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Japanese knotweed, *Fallopia japonica* is an invasive alien weed causing serious environmental and economical problems in Europe and North America. During field surveys in Japan, a rust fungus was observed in summer/autumn causing severe damage to the weed. This rust pathogen was identified as *Puccinia polygoni–amphibii* var. *tovariae* based on morphology. The rust was found from August to December in a field site at Kyushu University, Fukuoka Pref., Japan. Urediniospores were observed between August and October and were associated with severe damage and defoliation of Japanese knotweed followed by teliospores were occurred in the field from November to December. The symptoms appeared 5 days after inoculation (dai) and inoculated leaves were defoliated 15 dai. It has not been confirmed if *P. polygoni–amphibii* var. tovariae is autoecious or heteroecious. However, it may still have potential as a biological control agent for *F. japonica*, and further research is needed to elucidate its life cycle and host range.

INTRODUCTION

Fallopia japonica (Houtt.) Ronse Decr., commonly known as Japanese knotweed or "itadori", which is native to Japan, is regarded as a serious environmentally and economically important weed in its introduced ranges in Europe and North America. Although chemical and mechanical control are currently undertaken in the UK, these solutions are only effective in the short term (Djeddour *et al*., 2008). Therefore, more sustainable or long–term methods for management of this weed are considered.

Field surveys in Japan revealed that three diseases caused by two rusts and a leaf spot fungus were common (Kurose *et al*., 2008). Rust fungi have a very good track record in weed biological control tending to be highly co–evolved and specific to their hosts (Evans, 2002). Subsequently, one of the rust species was identified as *Aecidium polygoni–cuspidati* (syn. *Puccinia phragmitis*; Hiratsuka *et al*., 1992) (Kurose *et al*., 2009). However, since *A. polygoni–cuspidati* is heteroecious, this species is unsuitable as a classical biological control agent (Kurose *et al*., 2009). Attention therefore was focused on the other rust species which infected to Japanese knotweed and was particularly common in summer/autumn.

The aims of this present study are to evaluate the potential of this rust species as a classical biological control agent for management of *F. japonica* in the UK by investigating its taxonomy, epidemiology and biology.

MATERIALS AND METHODS

Collection of materials

Surveys were undertaken in Japan from 2003–2007 throughout the natural range of *F. japonica* and its close relatives on the islands of Honshu, Kyushu and Shikoku. Leaf samples with disease symptoms were collected from the field and placed in paper bags. Samples were dried in a plant press for 3–5 days, with daily changes of paper, and then transferred to wax packets and kept at room temperature.

Phenotypic characterization

For microscopic examination, spores were mounted in sterile distilled water (SDW), or 0.1% lactophenol in cotton blue on a glass slide (Ritchie, 2001). The samples were observed using an optical microscope (BX60F5, Olympus, Japan) fitted with a digital camera (C–2000Z, Olympus, Japan).

For scanning electron microscopy (SEM), fungal structures on fresh host tissue were placed in 2.5% glutaraldehide in 0.1 M phosphate buffer (pH 7.4). Samples were transferred to a bell jar attached to a vacuum evaporator, which was evacuated with a rotary pump until all were submerged. The fixed samples were washed in the buffer four times for 1h and postfixed in 2% osmium tetroxide in the same buffer for 1 h. The material was briefly rinsed in the buffer and dehydrated in a series of washes with 50%, 70%, 90%, 99.5% and up to 100% ethanol. After dehydration, the samples were transferred to *t*–butyl alcohol for three changes at 30 °C. The glass container containing the specimens in *t*–butyl

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alcohol was placed in a refrigerator at 4 °C (Inoue and Osatake, 1988). The *t*–butyl alcohol was then frozen within 10 min, and dried in freeze dryer (FD–1, Tokyo Rikakikai Co., Ltd., Japan) for 3 h. The dried samples were mounted with carbon tape onto a metal block and coated with gold using an ion sputter (JFC–1100E, JEOL, Japan). Samples were examined with a JEOL JSM–5200 scanning electron microscope linked to a camera (Mamiya Co., Ltd., Japan) at 25 kV accelerating voltage.

Disease development in the field

For observation of disease development of the rust on *F. japonica*, a field site at Kyushu University (Fukuoka Pref., Japan) was selected. Twenty plants were marked and the disease score was assessed every month as the mean disease score index using the following scale: 0=no symptom; 1=1–20% of infected leaves; 2=21–40% of infected leaves; 3=41–60% of infected leaves; 4=more than 61% of infected leaves and 5=infected leaves defoliated. Disease score was calculated using the following formula; Σ (number of infected leaves per rating × rating value) / total number of infected leaves.

Inoculation test

F. japonica from Omura (Nagasaki Pref., Japan) were used for inoculation tests using urediniospores of the rust collected from infected leaves originating from Kyushu University. Using a fine paint brush, these were mixed with talc (spores:talc=1:4) or with SDW containing 0.1% Tween 80, and then the suspension was applied to both leaf surfaces using a fine paintbrush. Inoculated plants were placed in a customized dew chamber set at 20 °C, without light for 48 h. Subsequently, all inoculated plants were maintained in greenhouse, together with inoculated controls. Ten replicate plants, with five leaves per plant, were used. This treatment was tested twice. Disease score as described above was recorded every three days.

RESULTS AND DISCUSSION

Morphology and identification

Most rusts have up to five spore stages including spermogonial, aecial, uredinial, telial and basidial stages. However, in the field, only three spore stages of the rust were observed, with no evidence of spermagonial or aecial stages. This rust caused severe damage with yellow/red chlorotic spots on the upper leaf surface (Fig. 1A, B) and defoliation.

Uredinia were found on lower leaf surface (Fig. 1C), occurring in autumn, then telia gradually replaced uredinia (Fig. 1D). Uredinia are initially sub–epidermal but swell and rupture the epidermis (Fig. 2A) then rupture and shed the dark chocolate–brown urediniospores (Fig. 2B). Each leaf can produce thousands of pustules, each one containing many thousands of asexual, repeatedly infective spores (Fig. 2A). These urediniospores are globose, ellipsoid or obovoid (Fig. 2B), and the size was measuring 20–27.5 × 20–22.5 μm (Table 1). Walls are echinulate, 1.2–1.5 μm wide, with 2 or sometimes 3, equatorial germ pores.

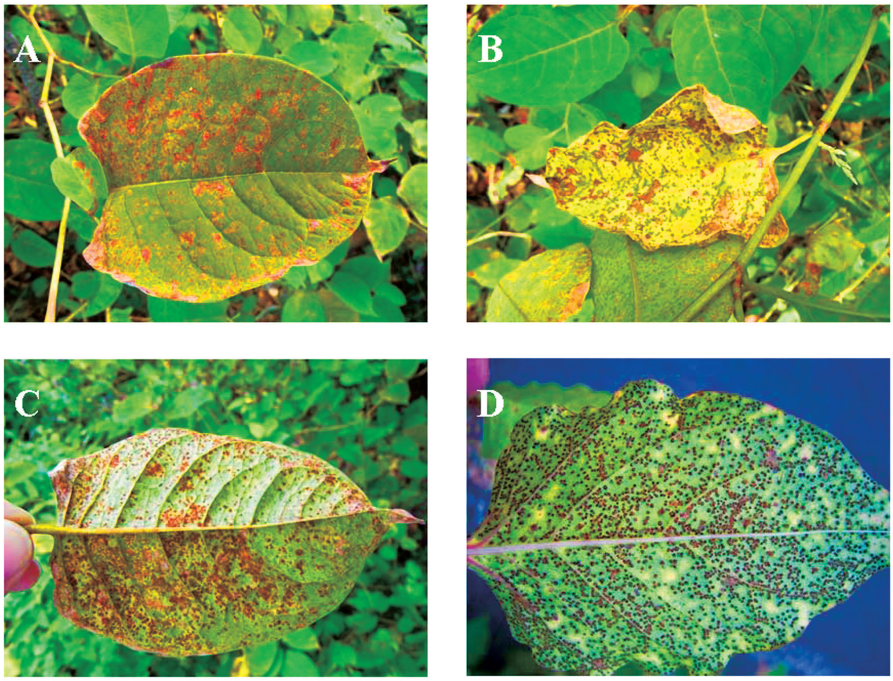

Fig. 1. Disease symptoms of *Fallopia japonica* infected with *Puccinia polygoni–amphibii* var. *tovariae* in the field. A; severe damage with yellow chlorotic spots and necrosis (taken at Kyushu University, Fukuoka Pref. on 18 September 2008), B; heavy infection and distorted leaves with premature senescence (taken at Kyushu University on 18 September 2008), C; uredinia (taken at Kyushu University on 18 September 2008) and D; telia, colored black, and uredinia mix (taken at Kyushu University on 27 November 2007).

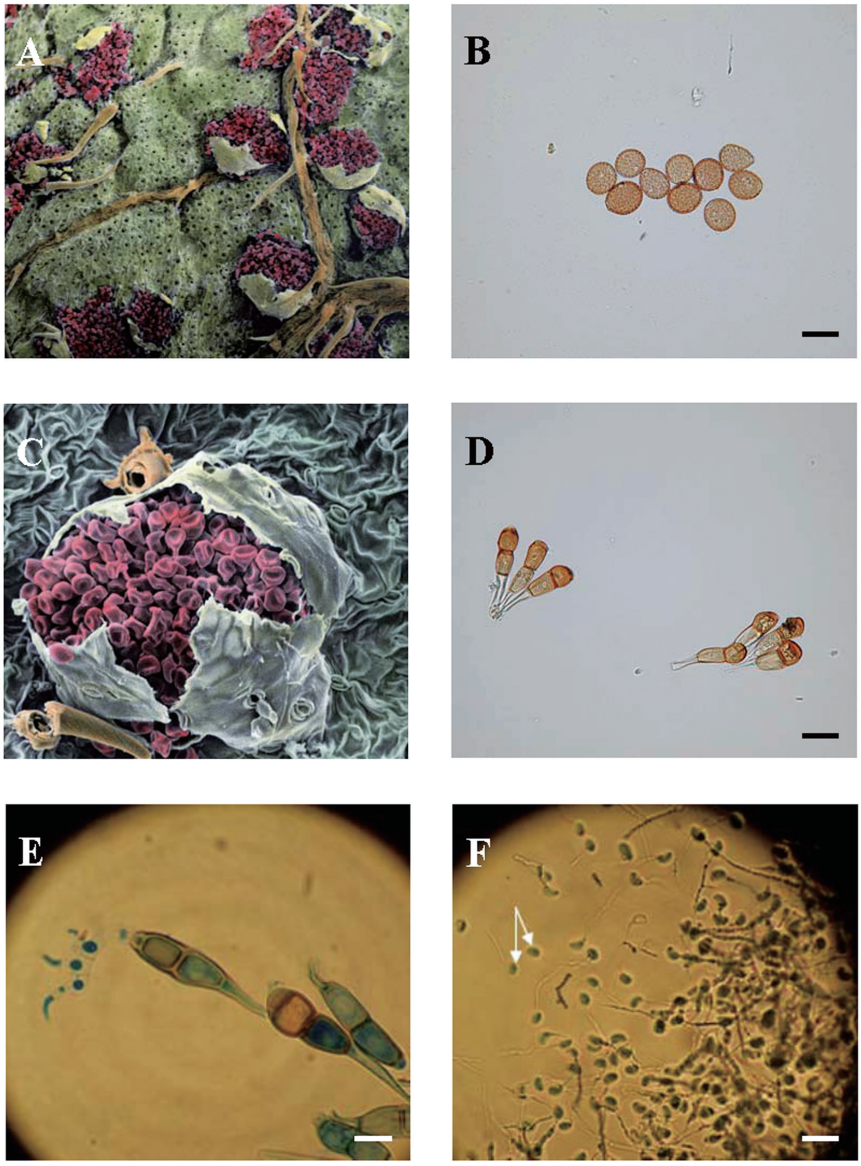

Fig. 2. Morphology of *Puccinia polygoni–amphibii* var. *tovariae*. A; uredinia by scanning electron microscope (SEM), B; urediniospores by optical microscope (OM), C; telium by SEM, D; teliospores by OM, E; germinated teliospore having borne four haploid basidiospores, one at the end of each sterigmatal tip by OM and F; bacidiospores by OM (arrow). Bars indicate = 30 μm.

Table 1. Morphological data of *Puccinia polygoni–amphibii* var. *tovariae* on *Fallopia japonica*

Specimen[a]	Locality of collection	Urediniospore			Teliospore		
		Shape	Color	Range (μm)	Shape	Color	Range (μm)
Original[b]	ND[c]	Globose, ellipsoid or obovoid. Pores 2, or 3, equatorial	Cinnamon–brown	18–30 × 16–23	Oblong to clavate, slightly constricted at septa	Yellowish brown	29–55 × 13–23
14–01–06	Mt.Hiko, Fukuoka Pref.	Globose, ellipsoid or obovoid. Pores 2, or 3, equatorial	Cinnamon–brown	18–28 × 15–22	Oblong to clavate, slightly constricted at septa	Yellowish brown	45–60 × 13–22
16–01–08	Kyushu University, Fukuoka Pref.	Globose, ellipsoid or obovoid. Pores 2, or 3, equatorial	Cinnamon–brown	20–27.5 × 20–22.5	Oblong to clavate, slightly constricted at septa	Yellowish brown	50–68.5 × 15–20
04–04–04	Unzen, Nagasaki Pref.	Globose, ellipsoid or obovoid. Pores 2, or 3, equatorial	Cinnamon–brown	19–30 × 17–23	Oblong to clavate, slightly constricted at septa	Yellowish brown	35–66 × 15–23

[a] Specimens were stocked in Kyushu University
[b] Data cited from Hiratsuka and Kaneko (1973)
[c] Not described

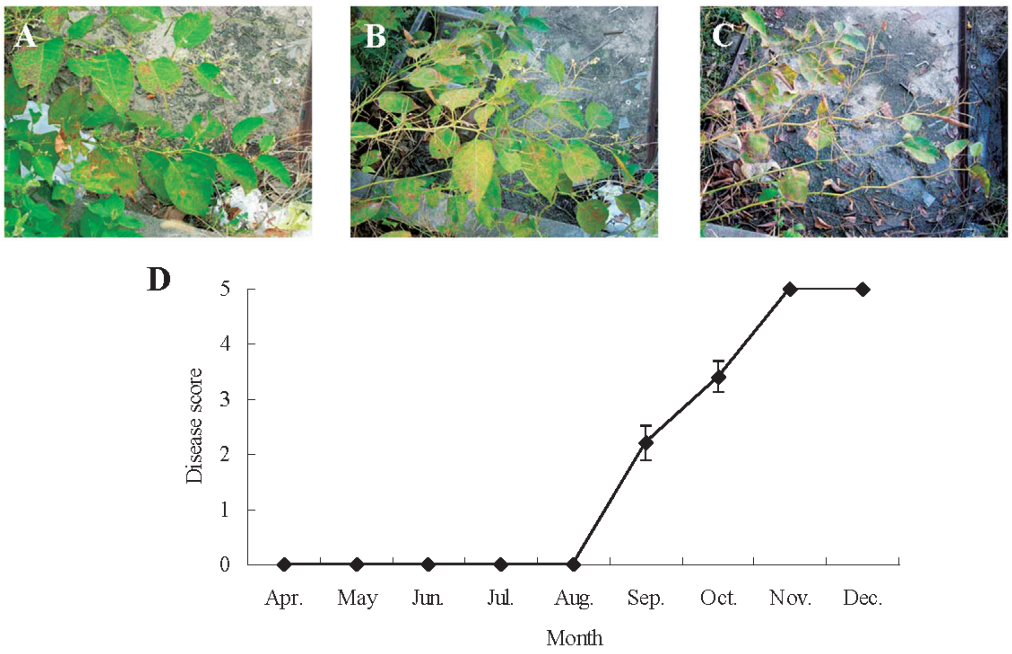

Fig. 3. Disease development of *Puccinia polygoni–amphibii* var. *tovariae* on *Fallopia japonica* at Kyushu University, Fukuoka Pref. on 2003. A; yellow/red chlorotic spots were started to appear (taken on 24 September 2003), B; heavy infection with yellow necrosis (taken on 21 October 2003), C; defoliation and some leaves with premature senescence (taken on 17 November 2003) and D; graphical representation of disease severity on *P. polygoni–amphibii* var. *tovariae*. Disease severity was assessed every month as the mean disease severity index using the following scale: 0=no symptom; 1=1~20% of leaves were infected; 2=21~40% of leaves were infected; 3=41~60% of leaves were infected; 4=> 61% of leaves were infected; 5=infected leaves all defoliated. Disease severity was calculated using the following formula; Σ (number of infected leaves per rating × rating value) / total number of infected leaves. Vertical bars represent standard error of mean.

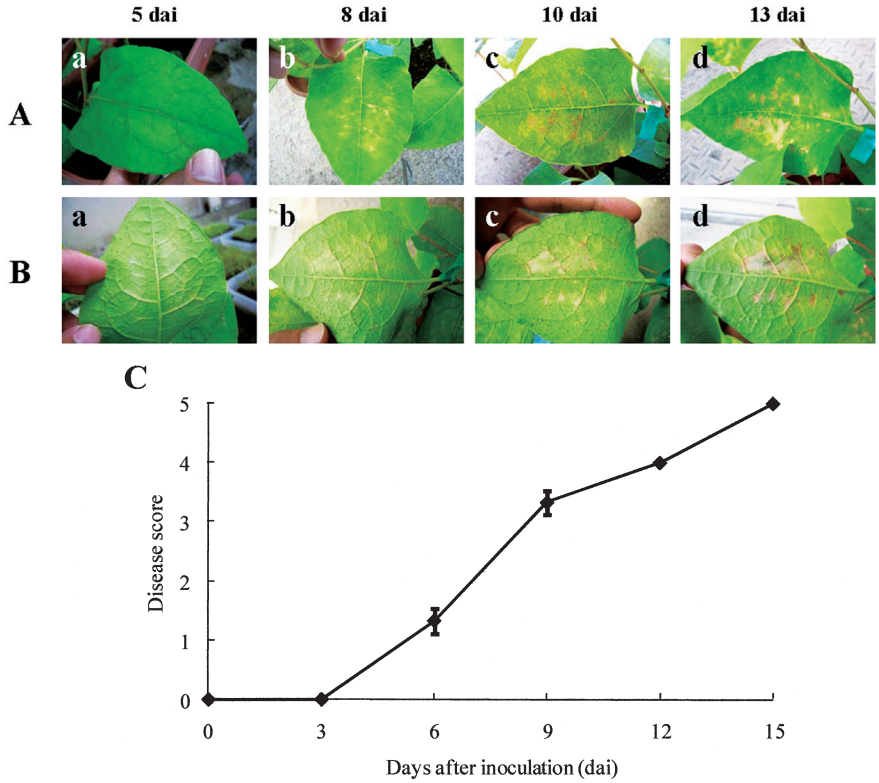

Fig. 4. Disease development of *Puccinia polygoni–amphibii* var. *tovariae* inoculated *Fallopia japonica* in the greenhouse. A; upper leaf, B; lower leaf and C; graphical representation of disease severity on *P. polygoni–amphibii* var. *tovariae*. Disease severity was assessed every three days as the mean disease severity index using the following scale: 0=no symptom; 1=1~20% of leaves were infected; 2=21~40% of leaves were infected; 3=41~60% of leaves were infected; 4=> 61% of leaves were infected; 5=infected leaves all defoliated. Disease severity was calculated using the following formula; Σ (number of infected leaves per rating × rating value) / total number of infected leaves. Vertical bars represent standard error of mean.

Telia developed towards the end of autumn and winter. They resemble a cushion or blister containing the two–celled teliospores on the underside of the leaf, and are darker in color and more resistant and compact than the uredinia (Fig. 2C). Teliospores are clavate and slightly constricted at the septa (Fig. 2D), measuring 50–68.5 × 15–20 μm. A germinated teliospore is shown in Fig. 2E bearing four haploid basidiospores at one of each sterigmatal tip. In Fig. 2F, the arrow points released basidiospores. As shown in Table 1, the rust was identified as *Puccinia polygoni–amphibii* var. *tovariae* according to the key and the comparison with the spore dimentions cited in Hiratsuka and Kaneko (1973).

Disease development

In the field

The incidence and development of the disease were observed at Kyushu University to help elucidate the life cycle of the rust. *P. polygoni–amphibii* var. *tovariae* was found in the field from August to December causing severe damage to all stages of *F. japonica* (Fig. 3A–D). The symptoms of yellow/red chlorotic spots started to appear towards the end of August and the beginning of September (Fig. 3A). Secondary infection by urediniospores was observed from September to October and caused severe damage and defoliation (Fig. 3B). In November, teliospores were observed on the lower leaf surface replacing urediniospores. All the diseased leaves were shed by December (Fig. 3D), although control plants were still alive and only became defoliated at January because of the cold temperatures.

In the greenhouse

Disease development of *P. polygoni–amphibii* var. *tovariae* inoculated *F. japonica* was tested in the greenhouse (Fig. 4A–C). Yellow chlorotic spots appeared on the upper leaf surfaces 5 days after inoculation (dai) (Fig. 4Aa); uredinia and pustules of powdery urediniospores were observed on the underside of leaves 8 dai (Fig. 4Bb). The inoculated leaves were shed 15 dai (Fig. 4C). Therefore, the inoculation test was demonstrated that the virulence of the urediniospores of *P. polygoni–amphibii* var. *tovariae* was high.

Mode of infection

Microscopic analysis of the infection mode by *P. polygoni–amphibii* var. *tovariae* using a scanning electron microscope showed that the inoculated urediniospores germinated, forming a germ tube with an appressorium over the stoma 3 dai. (Fig. 5A). At 8 dai, the uredinial pustule was formed under the lower leaf cuticle (Fig. 5B). In addition, thousands of urediniospores bursting from the lower leaf surface were observed 15 dai (Fig. 5C).

Life cycle

For *P. polygoni–amphibii* var. *tovariae*, uredinial, telial, and basidial stages were observed but spermogonial and aecial stages were not found in the field. These field observation confirmed the reports of Hiratsuka *et al.* (1992). The basidiospores failed to infect *F. japonica* and form spermogonia on the leaves. Field observations in this study were also inconclusive and did not allow us to determine whether *P. polygoni–amphibii* var. *tovariae* was autoecious, producing all of its spore forms on the same host species, or heteroecious, requiring two completely unrelated groups of host plants to complete the life cycle (Fig. 6). Some of the other closely related *Puccinia* varieties, for example, *P. polygoni–amphibii* var. *polygoni–sieboldii* Hirats. f. & S. Kaneko (Hiratsuka *et al.*, 1992) or *P. polygoni–amphibii* var.

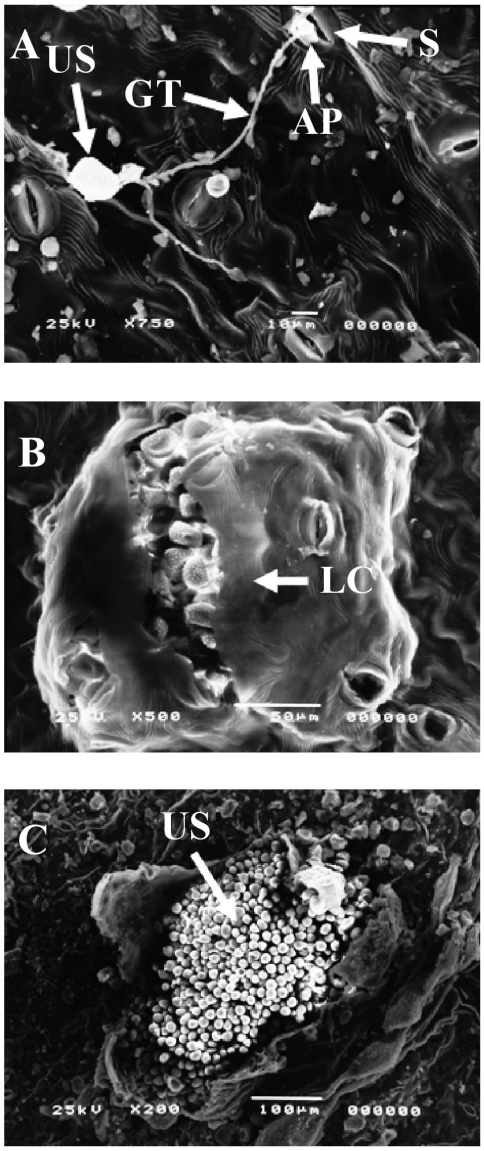

Fig. 5. Scanning electron micrograph of urediniospore of *Puccinia polygoni–amphibii* var. *tovariae* on the underside of leaf. A; germinating urediniospore, forming a germ tube with appressorium 3 days after inoculation (dai), B; formation of uredinium pustule 8 dai and C; thousands of urediniospores bursting from the lower leaf surface 15 dai. US; urediniospore, GT; germ tube, S; stoma, AP; appressorium, LC; lower leaf cuticle.

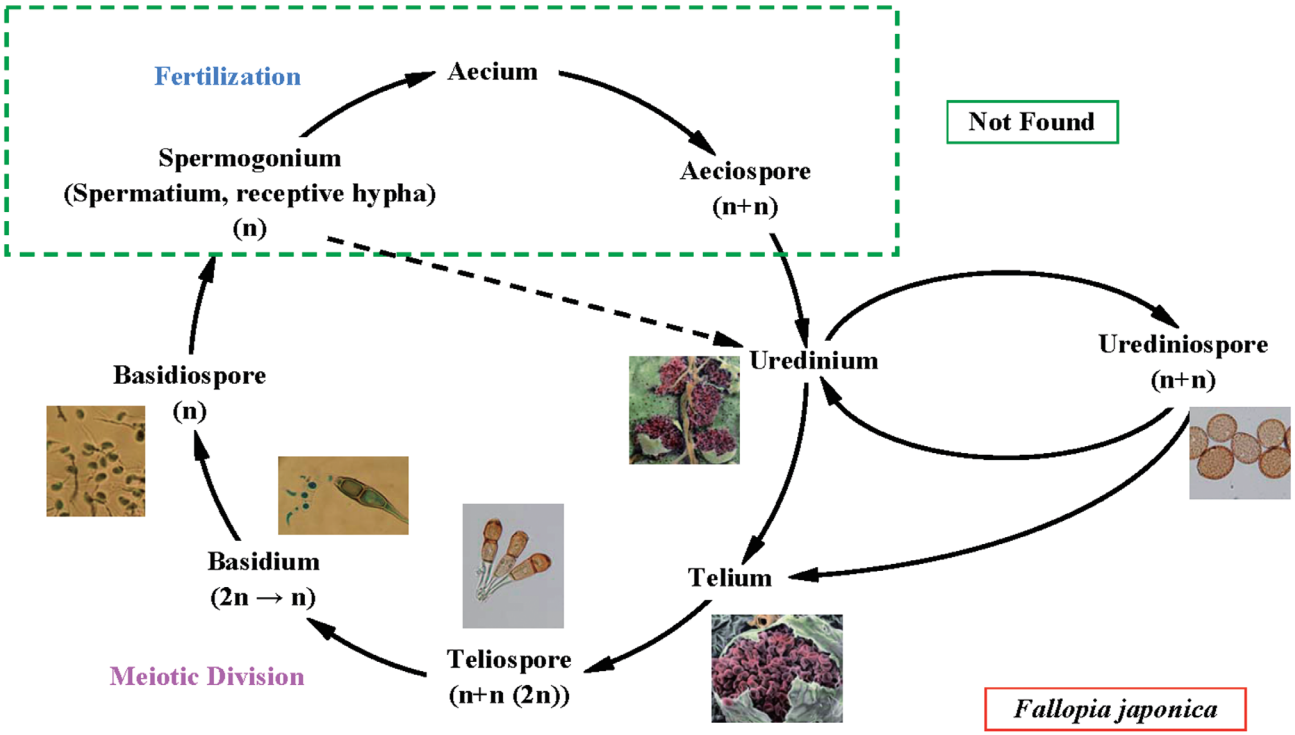

Fig. 6. Hypothetical life cycle of *Puccinia polygoni–amphibii* var. *tovariae*.

polygoni–amphibii (Hiratsuka *et al.*, 1992), have *Geranium* sp. reported as an alternative host.

Further research is needed to clarify the life cycle, specifically to test if *P. polygoni–amphibii* var. *tovariae* is infective only to *F. japonica*. Circumstantial evidence would suggest that it is heteroecious, although searches on adjacent plants, including *Geranium*, failed to find possible spermagonial or aecial stages. If this shows a positive result, it becomes a potential classical biological control agent for Japanese knotweed in the UK.

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