DOI 10.12905/0380.sydowia66(2)2014-0325

Exobasidium japonicum inhabits in node strategically during summer in *Rhododendron*

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Wang P.-H., Tsao C.-C. & Pai T.-Y. (2014) *Exobasidium japonicum* inhabits in node strategically during summer in *Rhododendron*. – Sydowia 66 (2): 325–334.

Exobasidium japonicum (Exobasidiales, Basidiomycota) is a fungus that causes the development of galls in tender tissues of azalea (*Rhododendron* spp.) from autumn to spring in Taiwan. It is not known where the pathogen inhabits during summer and how it infects the tender host tissues in autumn. Healthy shoots were infected by spores in disease season. In the offseason leaves and stems inhabited by the pathogen matured and matured leaves and stems did not cause galls. We sought to determine how the pathogen reaches young, developing tissues, which will develop several months later. We designed *Exobasidium*-specific PCR primers from rDNA ITS sequences of *E. japonicum*. These primers were used to locate the fungus in host tissues. *Exobasidium japonicum* was detected in the shoots with gall by PCR at the end of offseason and it was most frequently detected from nodal tissues by PCR. *Exobasidium japonicum* infected asymptomatic shoots, nearby the shoots with galls were detected as well. *Exobasidium japonicum* grew into petioles and inhabited the nodal tissue under the base of the petiole of infected leaves. The pathogen inhabits quiescently mainly in the nodes during the dry, hot season. From the meristem tissue of the nodes, it extends into axillary buds or adventitious buds when the nodes sprout. Depending on the organ that develops from the infected apical, axillary or adventitious buds, the galls are formed by tissue of leaf, stem, or flower organs. Thereby, *E. japonicum* is able to reach young, developing tissues and starts a new disease cycle. The PCR assay successfully detected low levels of *E. japonicum* DNA in *Rhododendron*, making this assay a good tool to study the ecology of the fungus.

Keywords: *Exobasidium japonicum*, inhabitant, PCR-detection, *Rhododendron*.

Woronin established the fungal genus *Exobasidium* in 1867, and most members of this genus infect plants and cause a hypertrophic enlargement of tissue of leaves, flowers, or stems of their hosts (Alexopoulos *et al*. 1996). Only few cases of systemic infection in the *Exobasidium* genus, such as *Exobasidium vaccinii* (Fuckel) Woronin produce a perennial mycelium in the rhizomes of its host, (Hilborn & Hyland 1956). *Exobasidium darwinii* M. Piątek & M. Lutz and *E. otanianum* var. *satsumense* X.Y. Zhang et K. Arai are

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partly systemic infections and affect shoots (Piątek *et al.* 2012, Nagao *et al.* 2001). *Exobasidium japonicum* Sharai causes Azalea leaf and stem gall, these galls being present throughout the year in temperate areas (Graafland 1960). Furthermore, gall formation has an optimum in certain months, most frequent during Belgian March and April months, when the flowering period is finished, and the plants are still kept in the greenhouse. During June and September, galls may be frequent on plants outside greenhouses (Graafland 1960).

The work of Graafland (1960), inoculating basidiospores of *E. japonicum* to the dormant axillary buds of *Rhododendron* showed the formation of galls on the shoots developed from the inoculated axillary buds after removing the tips of the inoculated shoots. The galls were formed from the buds that remained dormant for six months after inoculation. Based on his results, he proposed two infection pathways: i) the spores germinate between the hairs and scales on the surface of dormant buds and form viable colonies until the buds begin to expand; ii) the spores penetrate directly into the leaves of the dormant bud, and the development of the hyphae inside is delayed as long as the bud remains dormant. However, in subtropical areas of Taiwan, the disease is evident beginning mainly in October and ending in April (Shih 2006). From mid-May to September, the host vegetative growing season, the disease is not observed. The healthy shoots were infected by spores in disease season. In the offseason, the leaves and stems with the pathogen survived, matured, and matured leaves and stems did not cause galls. The aim of the present study was to determine how and where the pathogen inhabits during disease offseason and how it reaches the new-grown tender tissues which grow after the offseason.

In the laboratory, pure cultures of *E. japonicum* can be obtained by attaching infected tissues bearing basidia to the lid of a petri dish and allowing the basidiospores and secondary spores to fall onto potato dextrose agar or some other rich medium (Graafland 1952, Sundström 1964). Based on our experiences, *E. japonicum* is difficult to isolate from host tissue without spores, because it grows slowly and is frequently overgrown by saprotrophic fungi on culture media. Thus, it was important to establish a sensitive method to detect *E. japonicum* in host tissue. PCR-based techniques provide the opportunity to selectively amplify DNA sequences from an organism with a high level of sensitivity. The internal transcribe spacer (ITS) of the nuclear ribosomal RNA gene repeats, located on each side of the highly conserved 5.8S region, evolves rapidly and shows a high level of polymorphism. These features make it useful for studying variations among closely related species (White *et al.* 1990). The ITS region has been used extensively for the detection and identification of plant fungal pathogens (Wang & Chang 2003, Wang *et al.* 2003). For the present study, we developed a PCR-based method to detect the pathogen in Azalea plant samples, and to locate the fungus in Azalea plants.

Materials and methods

Study site and fungal isolation

Leaf and shoot galls formed by *Rhododendron mucronatum* (Blume) Don as a result of the infection by *Exobasidium japonicum* were collected on the campus of the Tunghai University, Taichung (N 24° 12', E 120° 36'), in central Taiwan. The morphology of basidia and basidiospores of *E. japonicum* was observed by light microscopy and the identification was confirmed by description of Nannfeldt (1981) and the analysis of rDNA ITS sequence.

The modified ballistospore-fall method (Derx 1930) was used to isolate *E. japonicum* from the galls. The galls with ripe hymenium on the surface were fixed inside the lid of a Petri dish. The basidiospores were ejected onto 3 % water agar, and the plate was kept at 24° C for 3 days, after which the germinated basidiospores and conidia were observed and isolated on potato dextrose agar (PDA, Difco, USA). Pure cultures were then preserved in glycerol and sterile water (1:1), and stored at –80 °C.

rDNA sequence analysis and primer design

The method of sterilization was modified from Guo *et al.* (2001). The leaves and stems were surface-sterilised by soaking for 3 min in a solution of 1 % sodium hypochlorite and then for 30 s in sterile water, blotted dry on sterile paper towels. Specimens of target tissues were cut aseptically and total DNA was extracted from *E. japonicum* isolates and plant tissues using a CTAB method (Doyle & Doyle 1990). Genomic DNA of *E. japonicum* WRS1-3 was amplified with the universal rDNA primers ITS1 and ITS4, as described by White *et al.* (1990). A 25 µl reaction mixture contained 1.0 µM of each primer, 1.0 unit *Taq* DNA polymerase (Fermentas, USA), 100 µM of each of the four deoxynucleotides, in a PCR buffer, and 0.25 µg of fungal DNA. The DNA target was amplified for 35 cycles of DNA denaturation at 94 °C (5 min for the first cycle and 1 min for subsequent cycles), 30 sec at 58 °C for primer annealing, and 1 min at 72 °C for DNA extension. PCR amplification containing no DNA template was carried out in every experiment as a control to test for the presence of contamination of reagents and reaction mixtures with non-sample DNA. The efficiency of amplification was monitored by running 5 µl of each reaction on a 1.2 % agarose (Amresco, USA) gel at 125 V in Tris-borate-EDTA buffer. A 100-bp molecular weight ladder (Pharmacia, Freiburg, Germany) was used as the size standard. The gel was stained with ethidium bromide and visualised and photographed under ultraviolet light. The rDNA ITS region was sequenced by Mission Biotech Corp., Taipei, Taiwan. Sequence data of *E. japonicum* WRS1-3 from this study was deposited in GenBank under accession no. GQ480730.

EMBL sequence accession AJ626911, AJ626915, AF297201, AF393431, and AB105234, representative of the ITS of diverse host *Rhododendron* species, were aligned with the same regions of *E. japonicum* WRS1-3 using

Clustal V. Primers were designed from the variable regions of the ITS of *E. japonicum*. To examine the specificity of the primers, Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information was used to search for species with sequences homologous to the primers from GenBank.

Specificity of PCR amplification

The optimal concentrations were examined in preliminary experiments to establish the reproducibility of the PCR amplification in order to maximise the yield. We tested annealing temperatures from 50–58 °C in 2 °C intervals, and the best annealing temperature for the sensitivity tests was chosen. The PCR cycling conditions were as follows: initial denaturation step, then 35 cycles of 94 °C for 60 s, 50–58 °C for 30 s, 72 °C for 60 s, and a final extension step of 72 °C for 10 min. Standard PCR and booster PCR were used to assess the sensitivity of the Exo-F and Exo-R primers for *E. japonicum*. Total DNA of *E. japonicum* WRS1-3 was serially diluted 10-fold from 10 ng to 1 fg and prepared as template DNA for PCR amplification. A 25-µl PCR mixture containing 25 ng DNA, 1.0 nM of each primer, 1.0 unit *Taq* DNA, and 100 µM dNTP was prepared in PCR reaction buffer for the first round of PCR. The amplification protocol consisted of a single denaturation step at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 sec, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. For the second PCR stage of booster PCR, 2.5 µl of the first PCR product was used as template in a 25-µl PCR mixture containing 1.0 µM of each primer, 1.0 unit *Taq* DNA, and 100 µM dNTP. Subsequent amplification reactions were performed using 35 cycles using identical reaction conditions as described for the first amplification.

Detection of *Exobasidium japonicum* from plant tissue by PCR

To investigate the dispersal and survival of *E. japonicum* outside of the disease season, shoots of *Rhododendron mucronatum* from four plots were collected in September, 2008. Each plot had 20 to 50 plants and many of them were diseased earlier in the spring. In each plot, two to three shoot samples with mummified galls and two to three neighbouring symptomless shoots were collected at random. A 1 cm long stem sample was collected every 5 cm both above and below the gall, or from the top of the symptomless shoot. Sample numbers were assigned depending on the length of the shoot. Three leaves were sampled from each shoot. To detect *E. japonicum* from plant tissues, DNA was extracted from 100 mg samples and amplified by booster PCR with *Exobasidium*-specific primers.

In order to investigate whether the pathogen survives in old stem tissues, we trimmed off the top 40 cm young shoots from the stems of the diseased plants in June 2009. Healthy plants grown in the greenhouse were treated as control. Axillary buds sprouted from the leaf scars of the top of

remaining stems in August, and the presence of *E. japonicum* in the buds, stems, leafs was investigated by booster PCR.

Field samples collected in August and September 2009 represent offseason samples. Samples collected from October, 2009 to April, 2010 represent disease season samples. To study where the pathogen survives during offseason, shoots with dried leaf gall or stem gall from individual trees were collected in September 2009 representing offseason samples. To survey the distribution of the fungus in the host tissue during disease season, we collected shoots with leaf galls from 11 and 8 plants in October 2009 and February 2010, respectively. DNA was extracted from tissues of leaf galls, leaves, petioles, leaf scars, stem galls and stem samples. The fungal DNA was detected by booster PCR.

Results and discussion

In this study, *E. japonicum* inducing tender tissue present in buds (Fig. 1 a, b), leaves (Fig. 1 c), flowers (Fig. 1e), and stems of fleshy gall-apples on *Rhododendron mucronatum* between autumn and spring in central Taiwan was observed. The galls were 1–4.5 cm in size, irregularly spherical, and

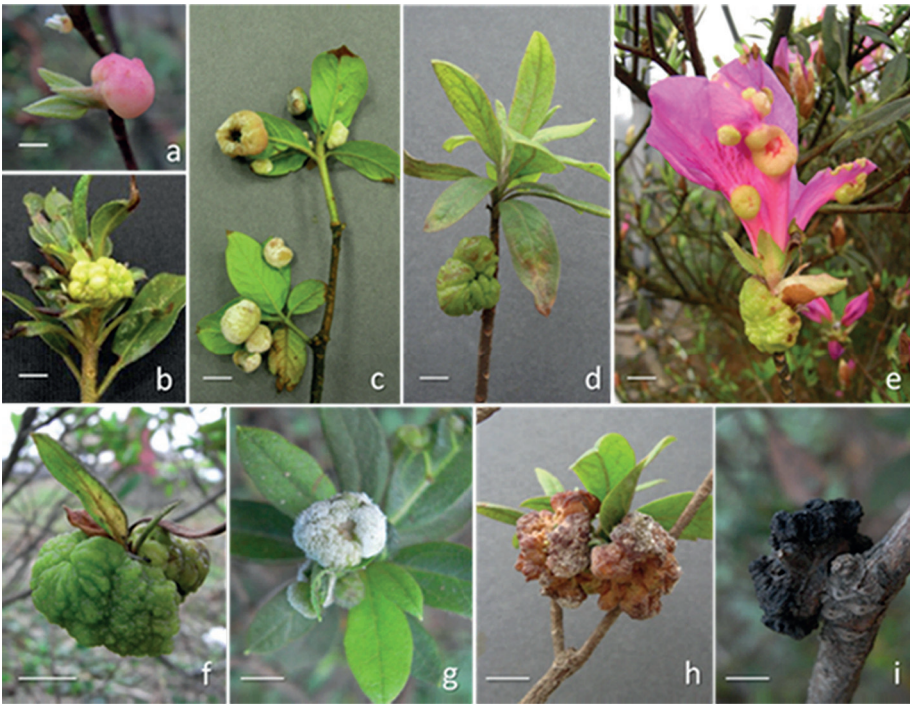

Fig. 1. *Rhododendron mucronatum* galls formed by: **a.** axillary bud, **b.** apical bud with witches' bloom, **c.** parts of leaves, **d.** tissue of stem, **e.** tissue of the flower, **f.** young gall, **g.** gall with sporulating fungal cells, **h.** withered gall, **i.** old, dry gall. Bars = 1 cm.

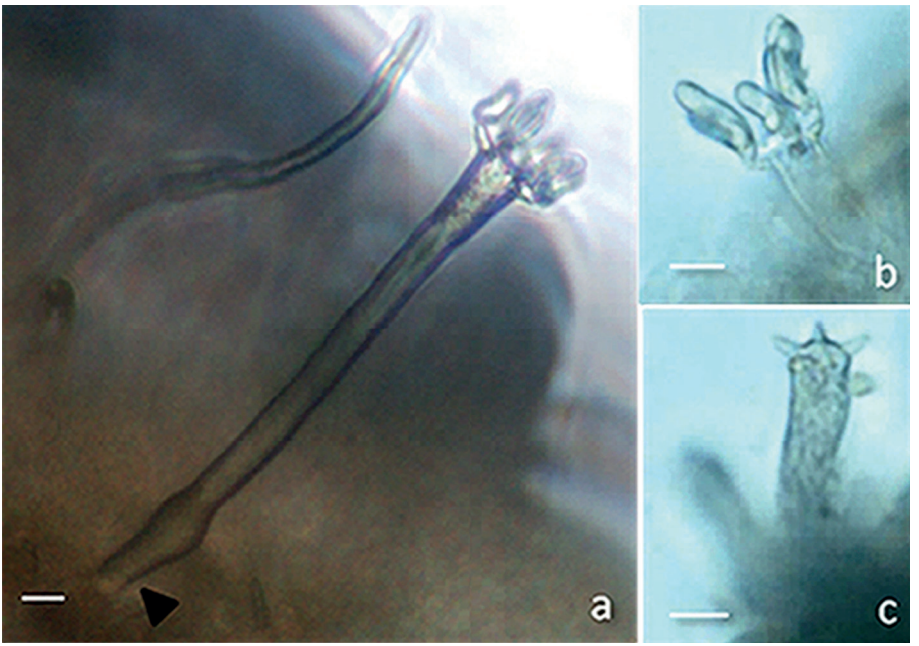

Fig. 2. Basidia and basidiospores of *Exobasidium japonicum* formed on the leaf gall of *Rhododendron mucronatum,* Bars = 5 µm. **a.** Cylindrical basidium with basal swelling and septum (arrowed). **b.** Basidiospores banana shaped, hyaline, smooth, one-celled when formed. **c.** Five sterigmata emerging outwardly.

greenish (Fig. 1 d, f) or yellowish green in colour (Fig. 1 b, c, e); sometimes they turned orange, pink (Fig. 1 a), or red. When they matured, the fungus formed hymenia, layers with white powder on the surface of the gall (Fig. 1 g). The base of the club-shaped holobasidia was distinctly swollen. Basidia bore three to six banana-shaped basidiospores on the top of subulate sterigmata (Fig. 2). Finally, the galls withered (Fig. 1 h) and dried, turned brown or black (Fig. 1 i), and either fell off or persisted on the plant in a mummified state. In some cases, the gall was developed by the apical bud, which formed witches' blooms (Fig. 1 b), and some diseased shoots wilted or dried. Infected azaleas showed symptoms of decline and dieback, causing the plants to die.

Nucleotide sequences of the rDNA ITS region of *E. japonicum* WRS1-3 were aligned with sequences from the same region from related *Exobasidium* species, and the primers Exo-F (5'- CAC CTG TGA ATC GTT GCT GAG -3') and Exo-R (5'- CCT TGG GCT TGC TTG CGG -3') were designed. Based on the sequence information, the primer pair was *Exobasidium* genus-specific, and the predicted amplification size of the *E. japonicum* product was 316 bp. Using optimised conditions (annealing temperature of 58 °C), the Exo-F/ Exo-R primer pair yielded an amplification product of the predicted size from *E. japonicum* WRS1-3. When a pure culture of *E. japonicum* was used

Tab. 1. Positive (+) and negative (–) results of detection of *Exobasidium japonicum* in stem and leaf tissue of shoots of *Rhododendron mucronatum* with mummified galls and nearby asymptomatic shoots of 20 plants in 4 plots (A–D) before disease season (September 2008) using booster PCR with the *Exobasidium* specific primer pair Exo-F and Exo-R.

Sample	Plot	Shoot code	Stem (cm)[a]								Leaf		PCR(+)/
			+10	+5	0	–5	–10	–15	–20	PCR(+)/ total stem no.	PCR(+)/ total leaf no.	PCR(+)/ total shoot no.	Total shoot no.
Symptomatic shoot	A	1	–	–	–	–					0/3		
	A	2		–	–	–					1/3		
	B	1			–	–	–				2/3		
	B	2			+	–	–				3/3		
	B	3			–	–	–	–		6/11	0/3	7/11	9/11
	C	1			–	–	–	+		(55 %)	0/2	(64 %)	(82 %)
	C	2			–	–	+	–			2/3		
	C	3	–	–	–	+	–				1/3		
	D	1	–	+	–	+					3/3		
	D	2			–	–	–				3/3		
	D	3			–	+	–				0/3		
Asymptomatic shoot close to symptomatic shoot	A	3			–	–	–	–			2/3		
	A	4			+	–	–				0/3		
	A	5			+	–	–				0/3		
	B	4			–	–	–				2/3		
	B	5			+	–	–	-	+	4/9	1/3	7/9	9/9
	C	4			–	+	–			(44 %)	1/3	(78 %)	(100 %)
	C	5			–	–	–	–	–		1/3		
	D	4			–	–	–				2/3		
	D	5			–	–	–				3/3		

[a] The gall location on the shoot as the start point (0 cm) of symptomatic shoot; the top of shoot as the start point (0 cm) of asymptomatic shoot. Stem sample number depending on the length of the shoot sample.

for DNA extraction and amplification, the minimum starting amount of DNA needed to detect the specific PCR product by ethidium bromide staining after 30 cycles of amplification was approximately 250 fg. Booster PCR (35/35 cycles) yielded detectable amplification products down to a concentration of 25 pg of DNA, indicating that this technique is highly sensitive and shows potential for pathogen detection.

Basidiospores and conidia were released from the galls caused by *E. japonicum* on *Rhododendron mucronatum* and infected nearby asymptomatic shoots in disease season. High detection rates were shown in both symptomatic shoot (82 %) and nearby asymptomatic shoots (100 %) (Tab. 1) at the end of offseason. The stem and leaf pathogen detection rates for symptomatic shoots were 55 % and 64 %, respectively. *Exobasidium japonicum* was most frequently detected in the shoot below the gall location. Before disease season (September), the detection rate in the petiole of the diseased leaves was 40 %, in the stem tissues close to attached diseased leaves was 33 %, and in the nodal tissues of the diseased leaves was 86 % (Tab. 2). To conclude from these results, the fungus mainly inhabited the nodal tissue under the base of the petiole of infected leaves. It quiescently inhabited these tissues and survived there for longer than six months as an endophyte through the dry, hot offseason.

Tab. 2. Booster PCR detection rate of *Exobasidium japonicum* from field-sampled tissues of *Rhododendron mucronatum* with leaf gall collected in offseason and disease season.

Date	No. of positive reaction /No. of samples tested (%)				
	Stem	Node	Petiole	Gall	Leaf
Sep 2009*	2/6 (33 %)	6/7 (86 %)	2/5 (40 %)		
Oct 2009	3/11 (27 %)	11/11 (100 %)	8/11 (73 %)	2/12 (17 %)	
Apr 2010	2/8 (25 %)	2/11 (18 %)	5/11 (45 %)	9/11 (82 %)	3/11 (27 %)

* offseason

On the other hand, the detection rate in both the stems and leaves of nearby asymptomatic shoots was 44 %. All nine asymptomatic shoots near the shoots with gall had been inhabited by *E. japonicum* in the stem or leaf tissue, and it was most frequently detected at the top of the shoot (Tab. 1). *Exobasidium japonicum*-associated disease occurred on the young, actively developing tissues, like corn smut (Agrios 2005). The fungus inhabited in mature leaves and stems did not cause galls. It grew into the petiole from infection sites, arrived at and inhabited nodal tissue strategically.

In October, 2009, nodal tissues at the galled leaf bases (100 %) and petiole tissues (73 %) demonstrated highest *E. japonicum* detection rate (Tab. 2). The pathogen inhabited in the nodal tissue extending into the buds (Fig. 3), thus it is able to grow into tender tissues and then starts the disease cycle.

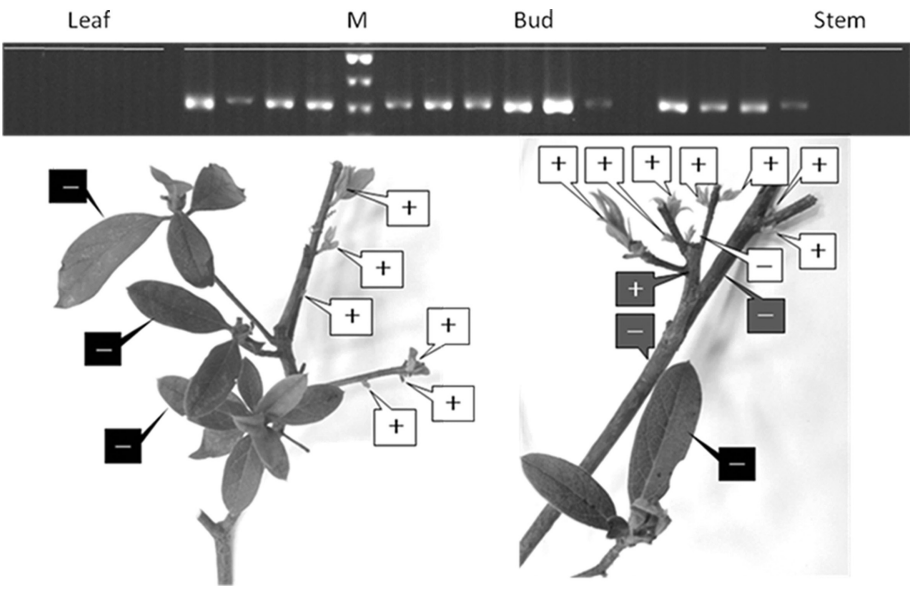

Fig. 3. *Exobasidium japonicum* was detected by booster PCR from buds sprouted from the stems of *Rhododendron mucronatum* which had been trimmed after disease season. The size of PCR product was about 320 bp. M: 100 bp marker, the lowest band is 300 bp. + : positive, – : negative reaction of PCR. White: bud; gray: stem; and black: leaf.

Depending on the organ that develops from infected apical, axillary or adventitious buds, galls form on the leaves, stems, or flowers. At this stage, galls showed the highest detection rate of *E. japonicum*.

In April, the end of the disease season, the fungus was detected in galls, leaves, petioles and the nodal tissues from the diseased leaves and in nearby stem tissues. The detection rate was 82 % for gall tissue and ranged from 18 % to 45 % for other tissues (Tab. 2).

To detect the survival of *E. japonicum* in old stems of *R. mucronatum*, the upper shoots of the diseased plants were cut in June, after the disease season. The remaining stems sprouted from leaf scars in six weeks (Fig. 3) but not from the node with leaves. *Exobasidium japonicum* was detected by booster PCR from 13 buds sprouted from leaf scars of the remaining stems but not from the control treatments. It was detected from the base of a small branch, but not from 2 thicker stem samples or matured leaves (Fig. 3). The axillary buds or adventitious buds that sprouted from the leaf scars of lower stems were infested after pruning during the offseason; those old stems developed at least a year ago (Fig. 3). This indicates that the pathogen inhabited not only in the tissues grown in that year, but it also survived in the lower, older woody stems. It still had the ability to infest new buds budding during the offseason.

Exobasidium japonicum inhabited several sites of the stem and was not systemic (Tab. 1, Fig. 3). It was limited in its capacity to spread throughout the stem. It invaded the stem through infected individual leaves, or the stem was developed from inhabited nodal meristem tissue.

The PCR assay successfully detected low levels of *E. japonicum* DNA in *Rhododendron*, making this assay a good tool to study the ecology of the fungus.

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(Manuscript accepted 29 September 2014; Corresponding Editor: I. Krisai-Greilhuber)