

# Identification of the fungus *Erysiphe diffusa* causing powdery mildew disease on soybeans in Vinh Phuc, Vietnam

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Received 16 November 2016; accepted 3 March 2017

## Abstract:

**Fungus *Erysiphe diffusa* Cooke & Peck 2000, which causes powdery mildew disease on soybeans (*Glycine max* (L) Merr) cultivated in Vinh Phuc Province, has already been identified using a combination of molecular biotechnology and an updated standard morphological classification for the confirmation of a pathogenicity test to fulfill Koch's postulate. Six sequences of the ITS rRNA region of this fungus on soybeans have already been registered in GenBank with accession numbers including KM260706 and KM260708 - KM260712.**

**Keywords: *Erysiphe diffusa*, pathogenicity test, powdery mildew, soybean.**

**Classification number: 3.1**

## Introduction

Vietnam is a tropical country with a monsoon climate characterized by heavy rainfall, high humidity, and hot temperatures. These are favourable conditions for the development and outbreak of various diseases on plants. Powdery mildew is one of the most severe diseases of these, often damaging and being found on many kinds of plant species belonging to various botanic families.

Around the world, powdery mildew disease is damaging areas used for cultivating soybeans, including Asia (China, Japan, Korea...) and America (USA, Argentina...). The disease became an epidemic in Korea, reducing approximately 65-70 percentage of the crop yield in many intensively cultivated areas [1]. In Oita Province, Kyushu Island, Japan, powdery mildew disease outbreak on soybean to epidemic-

extremes and caused a 35-40% reduction in yield [2].

The severe outbreak of powdery mildew disease is caused by a fungus belonging to the genus *Erysiphe*, and has been reportedly found on several soybean samples in parts of East Asia, including in Vietnam, since 1998 [3]. The formation of the sexual period (chasmothecia) is an important key to classify powdery mildew fungi. However, the taxonomic and phylogenetic positions of this fungus have not been recognized due to a lack of a sexual stage. In fact, it has been difficult to find out the formation of chasmothecia in tropical countries including Vietnam. Therefore, studies and the application of morphological classification based on the sexual stage of powdery mildew fungus have hardly been developed in tropical areas.

According to the statistics from the

General Statistics Office of Vietnam and Ministry of Agriculture and Rural Development, although the country of Vietnam is a great consumer market of soybeans, the acreage in Vietnam has had a decreasing trend from 181,000 to 120,000 ha, with an average yield of 14.3 ta/ha, which is in comparison with that of the world at 25 ta/ha. At present, soybeans are the main winter crops in regions with two annual rice seasons, like in Vinh Phuc Province. In 2014, Yen Lac was the district with the greatest soybean cultivation area in Vinh Phuc (1,128 ha), in which Lien Chau was the commune of 196 ha, concentrating on an area with a dyke. DT96, DT84 are popular soybean varieties cultivated with other local ones. Soybean cultivation has not only increased the income for farmers but also spread-out regions flush with intensively commercial productions, step by step and opening in the direction of changing crop structures sustainable for the winter season in Yen Lac District in particularly and Vinh Phuc Province in general. However, powdery mildew fungus has been reported as one of the major agents limiting acreage and productivity of soybeans in this locality. The disease has already damaged new varieties that have high yield such as DT26, DT84... [4].

To study the fundamentals of controlling powdery mildew disease effectively, the Ministry of Science and Technology of

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Vietnam has approved a Vietnam-Japan international co-operation project, titled the “Research and application of biotechnology for the classification and control of powdery mildew diseases of some crops in Vietnam” for the 2013-2015 period. This article outlines the latest study results for taxonomy of powdery mildew fungus on soybeans grown in Vinh Phuc, Vietnam ranging from the species level, based on a combination of molecular biotechnology and a standard morphological classification, which has been updated and implemented creatively during 2014 by the Plant Protection Research Institute (PPRI) [5].

### Materials and methods

*The place for gathering powdery mildew fungus samples:* The Tam Duong Commune of Tam Dao District, the Yen Lap Commune of Vinh Tuong District, and the Lien Chau Commune of Yen Lac District in Vinh Phuc Province.

*The preparation of the fungus source:* Powdery mildew fungus was isolated from disease samples found on soybeans characterized by symptoms on its medium leaves gathered in Vinh Phuc Province.

*The observation of morphology of powdery mildew fungus:* Conidiophore and conidia were extracted from powdery mildew disease infected soybean samples, which are still fresh, have been separated using transparent adhesive tape and then put onto lamen, and the number of conidia was counted and noted for its characteristics of conidiophore, foot cell, appressorium..., which was observed using a composed microscope at objective lens set at 40 X. In each sample, 100 conidia and 30-foot cells were measured for length and width.

The observation of the germination of conidia powdery mildew fungi was carried out according to the method of Hirata (1942) [6].

*Artificial inoculation:* In order to define plant pathogens, artificial inoculation was carried out under glass house conditions on the DT84 soybean variety, which was two weeks old and had one or two true leaves via press disease infected leaf to the fresh young one of 30 soybean plants. Meanwhile, 30 other freshly non-infected soybean plants were considered as negative controls.

*DNA extraction:* The total DNA genome of powdery mildew fungi was extracted from the conidia of six disease samples on soybeans of the Coc Nong local variety and DT84 popular ones which were gathered from Tam Duong Commune located in Tam Dao District, Vinh Phuc Province via a DNA Easy Blood and Tissue Kit (QIAGEN).

*Polymerase Chain Reaction (PCR):* In order to clone ITS rDNA regions, universal primer pairs HF1/HR4 were used according to the method of Tam, et al. (2015) [7]. The resulting product of the first PCR was used as the only template for the second PCR. The first PCR was carried out in a 0.5 ml tube with total 20 ml reaction volume, in which 0.5 µl was put into each primer (forward and reverse) at a preparation of 20 µM concentration, 10 µl Taq Polymerase Master Mix (TaKaRa, Tokyo), 4.0 µl dd H<sub>2</sub>O, and 5.0 µl gDNA of powdery mildew fungi. The reaction were run in a PCR thermal cycler SP (Takara, Kyoto, Japan) under thermal cycles containing the following steps: Denature at 94°C for 3 mins; followed by 30 small cycles, each including denature at 94°C for 30 secs, annealing at 58°C for 30 secs and extended at 72°C for 48 secs. The reaction was then finished at 72°C for 10 mins.

The second PCR was carried out in a total 30 µl reaction volume containing 20 µl Taq PCR Master Mix, 1.0 µl of each primer (forward and reversed at a concentration of 20 µM), 5.0 µl dd H<sub>2</sub>O, and 3.0 µl of the first PCR product at

gDNA template.

The PCR product was then isolated using 2% agarose gel electrophoresis with a 1% TAE buffer, presented as expected at band after submerging in 1% TAE containing Ethidium Bromide or Green Safe for 30 mins and then taking a photograph using UV sol gel machine. The band gel of the PCR product was cut and purified using QIAquick Gel ExtractionKit (QIAGEN) as described according to the manufacturer’s instructions.

*Sequencing and analysis:* Both directions of the DNA fragment were sequenced in order to analyze its ITS rDNA using the universal primer pairs HF1/HR4 mentioned above. The purified DNA fragment from the second PCR was then sent for sequencing at the First Base Co. (Malaysia/Singapore) or at Macrogen Co. (Korea).

Sequences at two directions were assembled using DNA star Lasergene 11 Core Suite software (<http://www.dnastar.com/t-allproducts.aspx>). Using BLAST, NCBI for searching sequences with the high identity of 98-100%. Then, using ClustalX Package [8] to align nucleotide sequences of powdery mildew fungi ITS rDNA was obtained in this study and compared to others in the DDBJ Database. After that, the alignment was seen and cut, or selected in MEGA5.2 [9].

Phylogenic trees obtained from analyzed data was found using method maximum parsimony (MP). MP analysis ran in PAUP 4.0 b10 [10]. Brands with bootstrap (BS) values were checked using BS analysis [11], and with 1,000 replications using optional conditions of stepwise addition previously ordered.

### Results and discussions

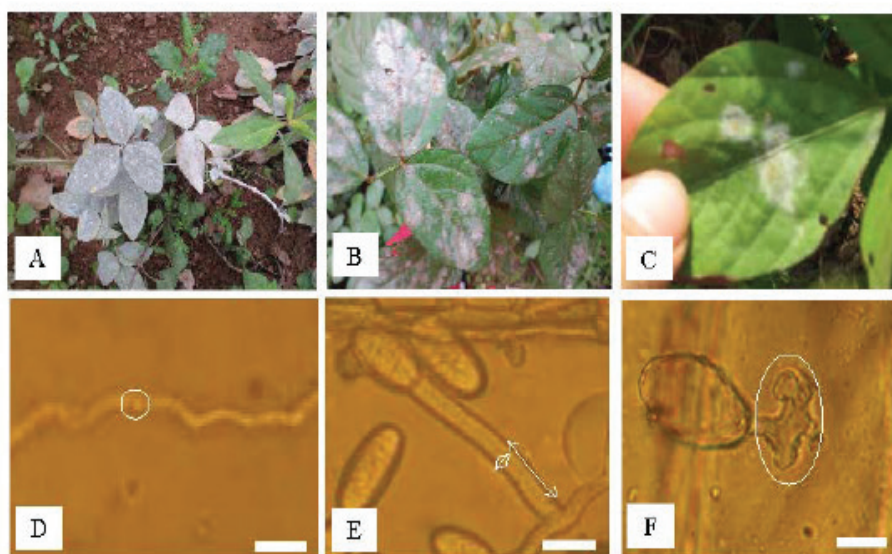
#### *Morphological characteristics of powdery mildew fungi damaging on soybeans in Vinh Phuc Province*

Powdery mildew fungi mainly

damaged medium and old leaves of soybeans. The first symptom that was present was white spots on both sides of the leaves, appearing after 1-2 weeks. These disease spots spread widely and merged together so that the whole leaf looks like it was covered by powdered lime. As observed under a composed microscope, the hypha of fungi mycelium had a sinuous form, and sometimes was present with big patches. Appressoria on hypha often had lobed or opposite lobed shapes, or multi-lobed shapes. Conidiophore often grew straight to its hypha. Conidiophore produced conidia singly with 1-2 dividing cells. Conidiophore had a length size measuring (43.3-)48.3-66.5(-71.5)  $\mu\text{m}$ . Footcell had a cylindrical form with a lengthxwidth size measuring (25-)27.5-35(-37.5) x 6.3-7.5(-8.8)  $\mu\text{m}$ . Conidia had a lengthxwidth size measuring (27.5-)30-40(-45) x (15-)17.5-20(-22.5)  $\mu\text{m}$  with lengthxwidth ratio calculated at (1.6-)1.8-2.0. Conidia had various kinds of shapes including cylindrical, egg, or oval shapes, but all without fibrosin. Conidia had a germination type of *Pseudoidium*. Chasmothecium had not yet been found. According to the morphological characteristics of anamorph stage (or unsexual stage) in comparison with the important classification keys which had been updated by Braun and Cook (2012) [5], powdery mildew fungus damaging on soybeans in Vinh Phuc, Vietnam belongs to *Erysiphe* genus (Fig. 1).

#### Artificial inoculation

Pathogenicity of powdery mildew fungi was defined using an artificial inoculation experiment and by pressing soybean leaves that were infected by powdery mildew onto 1-2 fresh young leaves of two-weeks old soybean plants of the DT84 variety, which is popularly cultured for production in real fields. Inoculation of powdery mildew fungi was performed on 30 fresh young



**Fig. 1. Several key morphological characteristics of powdery mildew fungi damage on soybeans in Vinh Phuc, Vietnam.**

(A, B, C) The symptoms of powdery mildew fungi were found on leaves of soybeans in glass house conditions in PPRI and on the fields of Vinh Phuc; (D) Hypha with a big patch shown highlighted by a white circle; (E) Conidiophore with length and width of foot-cell shown highlighted using white arrows; (F) Conidia germinated with *Pseudoidium* type shown highlighted by a white circle. The length of the bars is equal to (D, E) 30  $\mu\text{m}$  and (F) 15  $\mu\text{m}$  respectively.



**Fig. 2. Several photos illustrating experiments of powdery mildew fungi on soybeans in glass house conditions in PPRI.**

(A) An experiment of artificial inoculation; (B, C, D) Experiments into the sensitivity of powdery mildew fungi on soybean varieties (PPRI, 2014).

soybean plants. Meanwhile, 30 fresh others were used as a negative control (Fig. 2).

The plants were maintained in glass house conditions in PPRI from 26 to 28°C. Inoculated leaves developed typical symptoms after 10 days, while control plants didn't present any symptoms. The fungus presented on inoculated leaves had similar morphological characteristics to the one in the original source of inoculation.

Moreover, its sequence ITS rDNA from PCR product was 100% identical to the one in the original source of inoculation, fulfilling the demands of Koch postulate.

#### PCR for cloning ITS rDNA of powdery mildew fungi on soybean leaves

PCR results in order to define powdery mildew fungi on soybeans to species level are illustrated in Fig. 3 below.

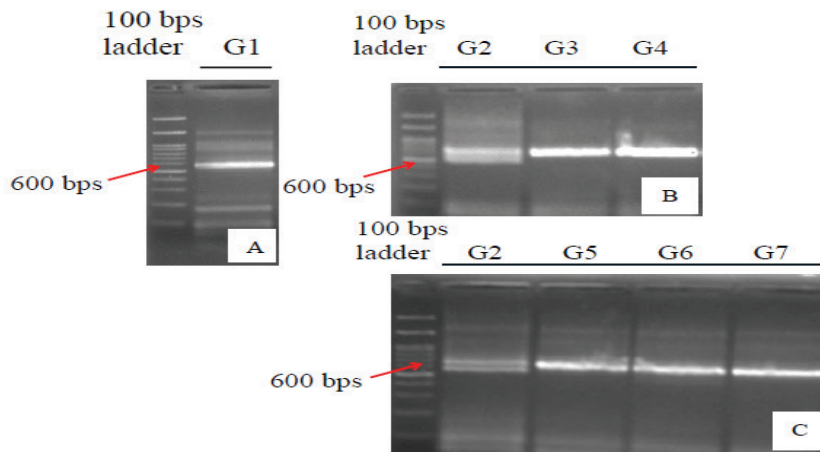


Fig. 3. Illustration containing PCR results for cloning ITS rDNA of powdery mildew fungi on soybeans in Vinh Phuc, Vietnam (PPRI, 2014).

**Material sources of powdery mildew fungi using for analysis phylogenic tree based on ITS rDNA**

Sequences of the ITS rDNA of powdery mildew fungi from six soybean samples has been registered with the GenBank Accession (GA.) from G1 and G3-G7, while others extracted are from the DNA Database.

**The establishment and analysis of the phylogenic tree MP based on the sequences of the ITS rDNA of powdery mildew fungi which damage soybeans in Vietnam by PAUP\* 4.0**

Six sequences of the ITS rDNA region of powdery mildew fungi on soybeans in Vinh Phuc, Vietnam were aligned with 17 sequences of the same genes of powdery mildew fungi belonging to the *Erysiphe* genus causing damage on other host plants obtained from the DNA Databases. Sequences of the ITS rDNA region of powdery mildew fungi *E. glycines* on *D. oxypHYllum*, GenBank Accession (GA.) AB015927, and *A. edgeworthii*, GA. AB015934 in Japan were used as an outgroup. The alignment sequences containing 23 taxa and 579 characters were observed to have 24 characters (equal to 4.15%) variable and 86 characters (equal to 14.85%) informative for phylogenic analysis. The analysis of the MP used PAUP, running

Heuristic searches and calculating the likelihoods of the results obtained, including transition/transversion = 2 (kappa = 4.0437887). Nucleotide frequencies were hypothesized as A = 0.19871, C = 0.27734, G = 0.27874, T = 0.24521. In 8,016 rearrangements, the

number of satisfied trees was two, and the score of the best tree was 133. The Kishino-Hasegawa test was used to find out the best tree; tree No. 1, which had the parameters of a tree length at 133, a consistency index (CI) at 0.9323, a retention index (RI) at 0.9692, a rescaled consistency index (RC) at 0.9036, and a homoplasy index (HI) at 0.0849. A heuristic search using a branch-swapping algorithm and Tree-Bisection-Reconnection (TBR) was carried out with 1,000 replications to establish a phylogenetic tree, in which its clusters would have bootstrap values similar to those represented in Fig. 4.

Analysis of the phylogenic tree based on the sequences of its ITS rDNA regions of powdery mildew fungi on soybeans (*G. max*) in Vinh Phuc, Vietnam, and around the world, as well as powdery mildew fungi of *Erysiphe* genus on other

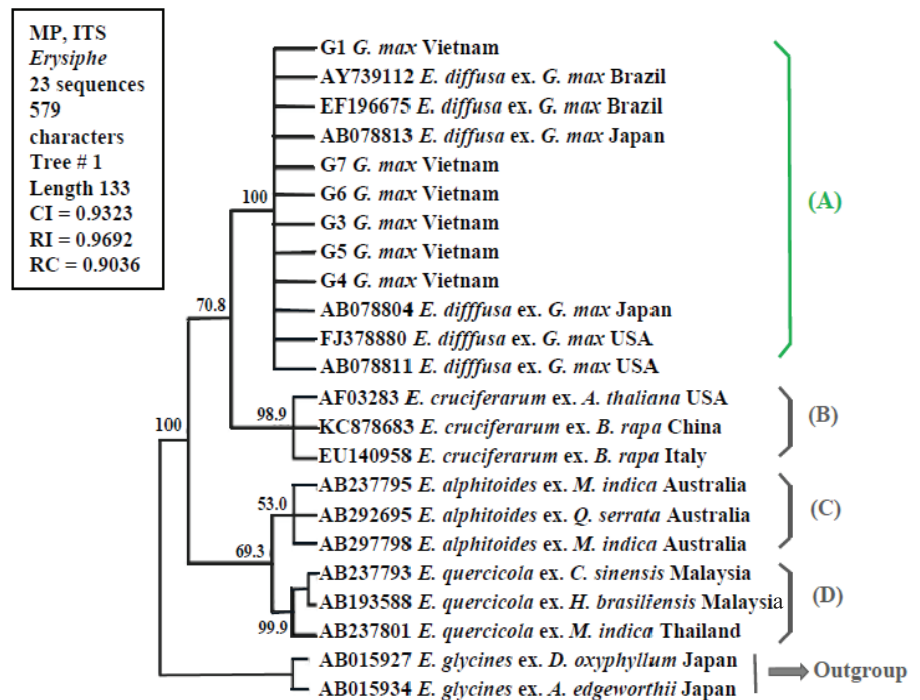


Fig. 4. Phylogenic tree MP, PAUP 4.0 based on analysis sequences of the ITS rDNA region of powdery mildew fungi on soybeans in Vinh Phuc, Vietnam and powdery mildew fungi on other host plants in the world.

Clusters (A) = *E. diffusa*; (B) = *E. cruciferarum*; (C) = *E. alphitoides*; (D) = *E. quercicola*; Outgroup = *E. glycines*. Bootstrap values calculated with 1,000 replications using optimal conditions of previously ordered stepwise addition were observed only presented at bootstrap values > 50%.

host plants, had already been shown that powdery mildew fungus was on soybean samples from Vinh Phuc, Vietnam; G1 and G3-7 along with more found on samples from Brazil, GA. AY739112, EF196675; 2 ones from America, GA. FJ378880, AB078811; and two more from Japan, GA. AB078813, AB078804, all belonging to cluster A, *Erysiphe diffusa* with 100 bootstrap value support.

From previous studies, *Erysiphe diffusa* Cooke & Peck, 2000 is synonymous with *Microsphaera diffusa* Cooke & Peck, 1872, and *Trichocladia diffusa* Cooke & Peck, 1927. This powdery mildew fungus has a host range including soybeans (*Glycine max*), French beans (*Phaseolus vulgaris*), and other plants with flowers from Fabaceae (*Alysicarpus longifolius*, *Apios americana*, *Crotalaria brevidens*, *Lespedeza bicolor*, *Lupinus perennis*, *Oxytropis campestris*...) and distributes in North America (Canada, Mexico, America), Middle and South America, Galapagos Archipelago, Asia (China, Korea, Japan, East USSR). *E. diffusa* could be divided into two lower levels of species based on morphological characteristics of appressorium on chasmothecium of teleomorph or sexual stage.

(1) *E. diffusa* var. *diffusa* has the length of appressoria 1.5-2.5 times longer than the diameter of chasmothecia. Its appressoria are shorter and rather stiff. This species often distributes on host plant genres including *Apios*, *Desmodium*, *Dolichos*, *Glycine*, *Glycyrrhiza*, *Lespedeza*, *Phaseolus*, *Senna*, and *Strophostyles*.

(2) *E. diffusa* var. *elongata* (Braun, comb. Et stat.nov.) MycoBank, No. 561423 has sinuous appressoria with their length of 2-4.5 times longer than the diameter of chasmothecia. This species often distributes onto host plant genres including: *Desmodium*, *Psoralea*, *Robinia*, and *Ruprechtia*.

However, reports of *E. diffusa* from Asia were uncertain of species found. The definition in the Asian reports

of *Lespedeza* was unclear. *E. diffusa* happened on a wide range of crops belonging to Fabaceae, and sometimes represented as a complex of several closely affiliated species, having allied taxa which were not separated yet [5].

For example, the molecular biological analysis of samples of powdery mildew fungi on soybeans gathered from Japan, Korea, Vietnam, and America showed that damage caused by the outbreak of the disease was a result of the combination of two species of *Erysiphe*. One species found at the sexual stage was the *Erysiphe glycines*. The other in Hanoi, Vietnam was uncertain because its sexual stage still had not been found yet, but it was suggested that it was likely an *E. diffusa*, the causative agent for powdery mildew fungi on soybeans in America considered so due to the identity of its ITS sequences. However, authors have not affirmed that it was exactly *E. diffusa* because there was an absence of its chasmothecia and, therefore, it was continuously asked to find out its sexual stage for accurate classification to species level [3]. Nowadays, the powdery mildew fungi can be fully classified at a species level, even without the observation of sexual stages. This is done using updated morphological characteristic keys of anamorph in combination with application biotechnology to sequence its ITS rDNA [5].

In this study, powdery mildew fungus in Vinh Phuc has morphological characteristics belonging to the *Erysiphe* genus, and has ITS rDNA identical to *E. diffusa* pulled from soybeans in Brazil, Japan, and America with a 100 bootstrap value supported in its phylogenetic tree MP which is being analyzed using specific gene software, PAUP\* 4.0.

Therefore, this paper does not need a present sexual stage, as it has already announced the first result in the definition of the powdery mildew fungus on soybeans in Vinh Phuc, Vietnam at the species level, *Erysiphe diffusa* Cooke & Peck, 2000 [12].

## Conclusions

The powdery mildew fungus which damages soybeans in Vinh Phuc, Vietnam has been defined as *Erysiphe diffusa* Cooke & Peck 2000.

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