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Studies on Morphological Characterization of *Erysiphe pisi* Causing Powdery Mildew of *Pisum sativum* by Environmental Scanning Electron Microscope

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ABSTRACT

Peas (Pisum sativum L.) is an important frost-hardy, cool-season, nutritious green leguminous pod-shaped vegetable belonging to family Fabaceae. Despite the importance of pea, the cultivation of crop is being hindered by several biotic and abiotic stresses. Among the biotic stresses, the disease causing pathogens are considered as the major factor the deterioration of quality and quantity of pea. With this, the powdery mildew disease caused by Erysiphe pisi is considered as one among the major disease of peas. Hence, there is a need to develop an effective disease management strategy. In order to develop an effective management strategy against the powdery mildew disease of peas the accurate identification and morphological characterization of fungal pathogen is essential. Till date very limited information's are available on the morphological characterization of E. pisi. ESEM analysis of E. pisi clearly reveal that the colour, shape and size of the conidia. The colour of the conidia was observed as hyaline. The shape of the conidia was varied from oblong (young) to Cylindrical (matured) conidia. The length of conidia was ranged from 24.84-33.06 µm with the mean of 29.09 µm whereas the width of the conidia ranged from 14.23-18.10 µm with the mean of 16.09 µm. Moreover, conidia harvested from P. sativum leaves were able to produce new powdery mildew conidia after 4-7 days of inoculation on healthy detached P. sativum leaves. This preliminary analysis of E. pisi provides information about its infectivity and morphological nature and it would helpful for better understanding of host-pathogen interaction, and may be useful in the development of early detection and effective management strategy in future.

Key words: *Conidia, Erysiphe pisi, Environmental scanning electron microscopy, Pisum sativum, Powdery mildew*

INTRODUCTION

The crop known as Peas (*Pisum sativum* L.) belongs to the *Fabaceae* family and is a native crop of Mediterranean region and Southwestern Asia[4, 15]. It has been considered as an important food legume worldwide after *Phaseolus vulgaris*[24]; however, this crop faces several cultivation problems, among which the harmful effects caused by fungi infections, especially powdery mildew, are the major restraint in obtaining expected yields.

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The causative of peas powdery mildew, *Erysiphe pisi* DC ex St. Amans. was recorded for first time in 1805 in Europe[13]. In 1851, the name of *Erysiphe martii* (Lév), technically refers only to an anamorphic fungal parasite[14]. In 1959, a new name for this species was published, *P. arthuriana*, then it was stated that the name of this species is *Ischnochaeta pisi* DC[19]. The *E. pisi* belonging to Erysiphales order has been reported in every part of the world as a global distribution wherever *P. sativum* is grown[6,23]. Erysiphales form an important group of plant pathogens that cause powdery mildew diseases in a diverse group of hosts nearly 10,000 species of angiosperms[1], which include cultivable crops and wild plants of economic and ecological importance in all kinds of temperate, arid, subarctic and tropical habitats[1]. These fungi have complex life cycles and are obligate biotrophs in nature. Because of these features, they are very difficult to study under laboratory conditions. Several studies have been conducted on this pathogen, all of which have focalised only on the morphological features of the fungus examined by light microscopy and all of them agree on the size and shape of reproductive structures [20, 21]. Major advantage of Scanning Electron Microscopy (SEM) over Light Microscopy, is that it allows us to reveal details of taxonomy, as well as various views of morphology such as surface particulars and parasitism and up to date has produced a wealth of knowledge on fungal pathogen and their host interactions[2]. Even though, the knowledge of this fungal group is still fragmentary[1], the identification of powdery mildews are depends largely on the teleomorph characteristics. This makes problems when a powdery mildew expands its host range or geographical location, because the teleomorph stage may not be seen for some years, or may not be produced at all[8]. An accurate identification is necessity in plant disease detection and in locating the source of inoculum. The identification may then be progressed beyond the broadly based *Oidium* type. The morphology and ultrastructure of *E. pisi* have already been studied by few workers by using light microscopy (LM), scanning electron microscopy (SEM), low vacuum scanning (LV-SEM) and transmission electron microscopy (TEM),[8,23]. Since there are no previous observations at environmental scanning electron microscopy study to reveal ultramicroscopic morphological characterization of the conidia of *E. pisi*, in this study we demonstrated a ESEM imaging of anamorphic conidia over the surface of pea leaf tissues, in order to obtain bi-dimensional view of them and truthful information for further studies on the interaction of *E. pisi* and *P. sativum* more precisely especially when such information is related with the known susceptibility of the host plant to particular powdery mildews. Studying biological samples with high water content needs specialized features like ESEM. The ESEM works under low vacuum and capable to image the living fungal propagules on their own host tissues without losing its original identity. Better understanding of morphological characterization of *E. pisi* would helpful to assess the variations and to develop an effective management strategy. However, the observation of *E. pisi* on live host through light microscope is very difficult because of close attachment with host and poor penetration of light sources. In this context, the present study was carried out to demonstrate the germination of conidia and assess the ultrastructural characterization of *E. pisi* using environmental scanning electron microscope.

MATERIALS AND METHODS

Collection and Maintenance of Inoculum

The field survey was conducted in major pea growing areas of Nilgiri district during 2015 - 16 and the powdery mildew infected leave samples were collected and brought to the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore - 03 for periodical maintenance. In order to maintain the field collected samples, the susceptible variety of peas (Arkel) was raised in pot culture. The pots were filled with farm yard manure, red earth and sand and five seeds per pot were sown. The pots were irrigated after

sowing and placed in glass house conditions. When the seedlings attained 20-25 days old at the four to eight node stages, conidia of field collected *E. pisi* were tapped on the leaves of potted plants. After 4-7 days white powdery mass was appeared on the inoculated leaves. Simultaneously, the potted plant inoculated with *E. pisi* produced maximum powdery mildew was considered as the virulent isolate of *E. pisi*. Such infected leaves were used as inoculum in later experiments such as detached leaf assay in moist chamber and also periodical maintenance under susceptible hosts.

Detached leaf assay for *E. pisi*

Detached leaf assay method was used to eliminate the other fungal contamination and to prepare the pure *E. pisi* infected leaf sample for conidial germination assessment and SEM analysis. The fungal spores of identified virulent *E. pisi* were harvested in 25-30 ml of sterilized distilled water (SDW) containing 0.05 per cent Tween 20 and the spore count of the stock suspension was estimated with an improved Neubauer haemocytometer. The spore concentration of the isolates adjusted to $1x10^5$ spore's ml^{-1}. The disease free healthy leaves of peas (Arkel) were collected from fresh potted plants and then the leaves were disinfected with one per cent sodium hypochlorite for one minute. The treated leaves were washed with deionized water containing 0.01% Tween 20 for three times. The inoculum (200µl) with conidial spore suspensions of $1x10^5$ spores ml^{-1} was applied on the abaxial surfaces of disinfected pea leaves. Finally the inoculation of test material was done using a settling tower to give a density of about 5 conidia/ mm^2. After inoculation petri dishes containing 6 ml of 5% sucrose moist sterile filter paper were wrapped and incubated in growth chamber (HECO environment chamber) at day/night temperature of 20±2°C/16±2°C under a photoperiod of 16 h light and 8 h dark and 85% relative humidity in plant growth chamber [25]. The germination of powdery mildew conidia on leaves was demonstrated under microscopic image analyser for further morphometric measurement through scanning electron microscope

Germination of conidia

The germination percentage of *E. pisi* conidia on the artificially inoculated pea leaves was assessed. With the aid of a sterile needle, spores were collected from incubated leaves with symptoms of powdery mildew and placed in microcentrifuge tubes (1 mg/ tube) having 1 mL of deionized water containing 0.01% Tween 20 and they were then allowed to germinate at 20±2°C with a 12 h photoperiod. Spore germination was assessed at 12, 24, 36, 48, 72 and 96 hours. For each assessment, the spore suspension was mixed and three subsamples of 20µL were removed from the microcentrifuge tubes and examined with a light microscopic image analyser (LaboMet) at 40X magnification. The fungus genus and species was identified with the use of specialized identification keys[20].

Environmental scanning electron microscopy (ESEM) analysis

The samples from the detached leaf assay method were used for further morphometric analysis using SEM analysis. The morphometric analyses of selected powdery mildew infected specimens were examined by ESEM (FEI-Quanta 250) using xT microscope control software available at the Department of Nano Science and Technology, Tamil Nadu Agricultural University, Coimbatore. This instrument, which has a specialized environmental safety mode prevent the desiccation of live specimen against high vacuum pressure on the stage and several fold increase in resolution over that of the standard light microscope and an exceptional depth of field, is currently being used to depict the surface characters of biotic agents without any fixation, dehydration and coating steps. For this purpose, leaf material with powdery mildew fungi was hand cut with a razor blade into small pieces of approximately 5 x 5 mm. The live specimens were directly fixed in carbon stubs and ESEM analysis was carried out using an acceleration voltage of 8-10kV. Image capture control was achieved using Auto-Montage v.5.0 (Synoptics) and the

images were captured as a series of focal planes and montage to produce a composite focused image.

RESULTS

The survey of the present study observed the maximum incidence of powdery mildew disease in pea growing areas of Nilgiri district, Tamil Nadu. Among the different cropping period, the winter season of August to January was observed the maximum incidence of powdery mildew disease than in other. Subsequently, the powdery mildew sample was collected and observed their symptoms as small irregular areas of powdery white mildew on the upper leaf surface of the lowest leaves and stipules were collected. The lesions spread rapidly up the plant and the stem becomes completely covered by the filmy white growth. Pods then become infected and the fungus was found to produce small black fruiting structures within the lesions (Fig. 1).

Morphological characterization

The light microscopy observations of the present study demonstrated that the conidiophores of *E. pisi* are hyaline, thin-walled and chains of conidia which are hyaline, thin-walled and oblong conidia. The length and width of the conidia were recorded as 25.4 to 45.4 µm and 10.5 to 18.6 µm with the mean of 36.5 and 15.0 µm, respectively. Whereas the length and width of conidiophore were ranged from 34.7 to 26.0 µm and 8 to 10 µm with the mean of 91.4 and 8.7 µm, respectively.

Germination of *E. pisi* conidia in a detached leaf assay

Detached pea leaves were able to remain as such up to 10 days under incubation in the detached leaf assay experiment. Conidia collected from the infected leaves retained their infectivity and were able to produce new colonies when inoculated onto healthy detached pea leaves under controlled environmental conditions. Seven days after inoculation, colonies were observed on 50 per cent of the inoculated leaves and *E. pisi* conidia were recovered from the leaves (Fig. 2). Conidia of *E. pisi* were observed on the flooded slides at 40x magnification. With this the maximum of 43 conidia and minimum of 13 conidia were recorded in a haemocytometer field. After artificial incubation, few conidia appeared to germinate and produce large lobed primary appresoria, which were bulbose, sometimes with small, rounded protuberrances and then to develop several hyphae radiating out across the host epidermis. Conidia were not found collapse even after germination (Fig. 3).

Environmental scanning electron microscopy analysis of *E. pisi* spore infectivity

In order to assess the detialed morphometry of the *E. pisi* the Environmental Scanning Electron Microscopy (ESEM) analysis was carried out with the direct vacuum on it. The result of the morphometry analysis with ESEM images showed that the length of the conidia ranged from 26.66 to 32.69 µm with the mean of 29.09 µm whereas the width ranged from14.23 to 15.60 µm with the mean of 16.09 µm. In addition, the structure of the conidia was also observed and showed that the surfaces of hyphae were usually smooth, but observations at high vacuum showed that the hyphae were shrinking with fibrillar material and some cells were verrucose. Ungerminated young conidia of *E. pisi* were also observed, usually singly but sometimes in short chains lying on the epidermis of *P. sativum* leaf. The average size of young conidia was ranging from 24-33 µm in length and 14-18 µm in width (Fig. 4 & 5). Thus, the conidial morphometry and surface colonization over the pea leaf was observed distinctly in this study.

DISCUSSION

The identification of fungal pathogens and its characterizations are crucial for the development of effective management strategy. Studies have been resulted the identification and morphological structures through visual or light microscope with limited information. With this the Environmental Scanning Electron Microscope (ESEM) plays a major role for the accurate identification and

morphological characterization of fungal pathogens. Thus, the present study was carried to assess the morphological characterization of *E. pisi* through ESEM. Initially, the survey was conducted the distribution of powdery mildew distributions in major peas growing area of Nilgiri district Tamil Nadu and the results demonstrated that all the surveyed areas recorded the maximum incidence of powdery mildew disease in peas during winter months of August to January. The result of the present was in accordance with the earlier report [18] they reported that the maximum incidence of powdery mildew disease in major peas growing area of Nilgiri district during the cooler period. The present results of detached leaf assay indicated that *E. pisi* has several adaptations that permit rapid and efficient colonization of pea leaf surfaces. Much of the literatures relating to the development of *E. pisi* have focused on the later stages of *E. pisi* development, following hyphal colonization on the host surface and the formation of conidia[22, 29]. Already, studied the *E. pisi* for formation of germtubes on natural infection, but did not give a detailed account of appresorial or hyphal development. *E. pisi* is an obligate pathogen, it is very difficult to maintain the specimens in order to work, for example, on the genetic improvement for powdery mildew resistance of *P. sativum*, through artificial inoculations [12]. In this sense, the possibility of having viable spores for a considerable period of time is very important. The detached leaf assay which has been used in culturing powdery pathogens provides a method that overcomes the time and space limitations of greenhouse and field evaluations[3]. A critical aspect in using the detached leaf assay is to maintain healthy leaf tissue for the period of time that is required for disease development. This test proved the usefulness of this technique, not only for determining spore infectivity in a short time but also in maintaining live and other fungi contamination-free *E. pisi* spores under *in vitro* conditions. Since it has been reported that powdery mildew may be parasitized by several hyperparasites[11]. Scanning electron microscopy of fungi has shown that although the cytoplasmic components are essentially similar to those of other organisms, their distribution is characteristic of each particular part of a hypha or mycelium and reflects the function of that microregion. The conidial apparatus in the Erysiphales offers a model system for ultrastructural physiology because, conidia arise following extension of one cell which divides to form a conidium initial which matures and a new generative cell which repeats the process[26]. Thus in some respects the physiology and therefore the ultrastructure, of the generative cell may be expected to possess features of a hyphal apex whereas maturing conidia undergo a progressive differentiation. In this study, we found that the *E. pisi* live specimens obtained from different farmer's field, when examined under light microscope and ESEM, showed reproductive structures and ranges of spore dimensions similar to those described in previous studies of *E. pisi* in *P. sativum.* Additionally, the present study also examined the morphological features of this powdery mildew through environmental scanning electron microscopy (ESEM) for the first time, and we are able to provide clear evidence of a cross section of a *P. sativum* leaf with mycelia, conidiophore and conidia. The ultrastructure of conidium of *E. pisi* agrees with the morphological keys[7, 17, 26] and results of electron microscopy characters of *E. pisi* was in accorded with earlier work [16]. In general individual cell types of this organism are strikingly different from one another and in each chain the conidia initials represent successive stages in a sequence of differentiation[16]. Other methods of preparation of SEM have been used for *E. pisi*, but results achieved seem to have been less satisfactory than those obtained with frozen, hydrated samples in this study. Fixed and desiccated material of *E. pisi* was considerably distorted under high vacuum pressure[10]. Furthermore, the ESEM images clearly had shown the presence of conidia on a *P. sativum* leaf.

Fig. 1: Symptoms of peas powdery mildew

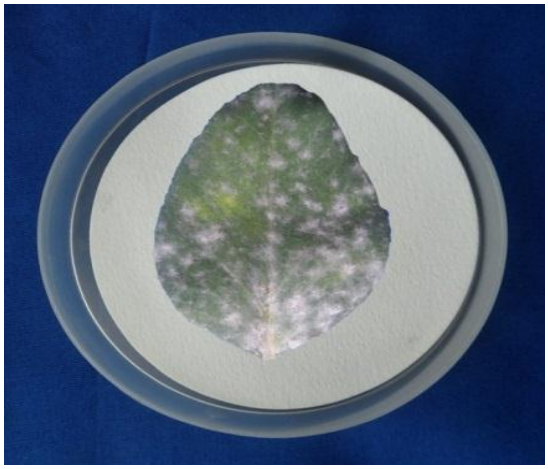

Fig. 2: Detached leaf assay

Fig. 3: Formation of germtube and appresorium on *E. pisi* conidia

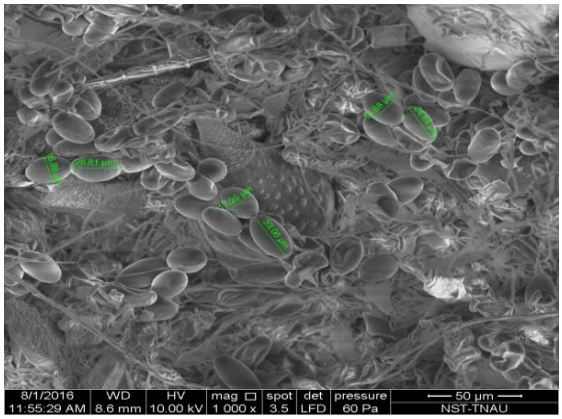

Fig. 4: Scanning electron micrographs of *E. pisi* propagules on leaf surface

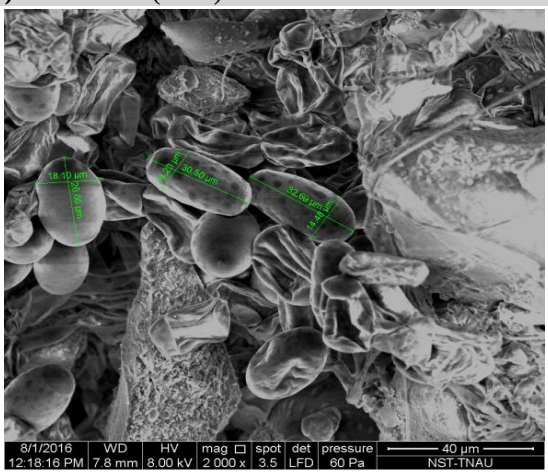

Fig. 5: Scanning electron micrographs of *E. pisi* conidia on leaf surface

CONCLUSION

The findings of this study provide new information on the morphology and biology of this host-pathogen interaction, and will serve as the basis for further studies on early detection tools, planning precautionary measures and development of effective management strategy.

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