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MORPHO-MOLECULAR IDENTIFICATION OF POTATO SILVER SCURF CAUSED BY *HELMINTHOSPORIUM SOLANI* AND STANDARDIZATION OF SPORE INUNDATION TECHNIQUE

Rahul Kumar Tiwari[a,b], Sanjeev Sharma[a], Ravinder Kumar[a], Milan Kumar Lal[a,b], Kailash Chandra Naga[a], Dharmendra Kumar[a], Vinay Sagar[a]

ABSTRACT: Potato is considered as a future crop to secure food security in developing nations. Of many storage diseases that impede the trade value of potatoes, silver scurf is steadily rising. The causal fungus *Helminthosporium solani*, belongs to *Ascomycetes* group and it attacks the potato tubers in the field as well as in storage. During the year 2019 the tuber samples were obtained from cold stores of Madhya Pradesh which were symptomatic to silver scurf disease. The appearance of tuber comprised of silvery skin blemishes on the periderm of tubers. The inundation of spores was standardized from the silvery lesions by incubation of infected tubers at different temperatures (12, 18, 24 °C). The spores were observed in the form of whorls on the tuber surface and these whorls were also visualized in compound microscope using double adhesive tape method. The pure culture of fungus produced initial hyaline mycelium which turned blackish brown after 21 days. The fungus was observed as extremely slow in its growth. The conidiophores were septate having a thin apex and broader base and melanized conidia were observed in a basipetal succession. The conidia were recorded to have two to eight pseudosepta. The molecular identification of fungus was performed through internal transcribed region based universal primers. The obtained sequences were submitted to the NCBI *GenBank*. An attention towards the pathogenomics, infection process and management strategies of silver scurf disease is mandatory to strengthen the processing industries involved in potato production system worldwide.

Keywords: Silver scurf; *Helminthosporium solani*; Mycelium

INTRODUCTION

As per the estimation of global consumption rate, the potato (Solanum tuberosum L.) comes first among the noncereal food crops (Devaux *et al*., 2019; Kumar *et al*., 2019, 2020; Lal *et al*., 2020a, 2020c, 2020b; Tiwari *et al*., 2020b). Moreover, the potato crop is also designated as future vegetable crop to ensure food security since 74% of total produce is utilized for consumption. The main reason behind such widespread importance is the short maturity period, diverse cultivation pattern, high calorific value and nutrition index of this wonder crop (Lal *et al*., 2021a). The leading vegetable crop occupies 19.23-million-hectare area with a global annual production of 390 million tonnes (Kumar *et al*., 2021). India comes after China in potato production and potato is grown on 2.13 million hectares with production and productivity of 44 million tonnes and 20.5 tonnes per hectare respectively (Kumar *et al*. 2019; Kumar *et al*., 2021). There is a consistent focus to increase the productivity of this crop but at the same time minimizing losses due to biotic and abiotic stresses is also critical (Tiwari *et al*., 2020a, 2020b). The biotic stressors such as fungi, viruses and bacteria are consistent threat in potato production system (Kumar *et al*., 2020d, 2021c). The late blight of potato

[a]ICAR-Central Potato Research Institute, Shimla-171001
[b]ICAR-Indian Agricultural Research Institute, New Delhi-110012

which was the major cause of historical devastation on potato crop, still troubles the potato growers (Sharma *et al*., 2021). Likewise, there are more than 40 viruses which hampers the potato production worldwide (Naga et al., 2019). In addition to that, with globalization and trade and changing consumer preference the occurrence of storage rots such as soft rot, dry rot, silver scurf, black scurf and other blemishes are also emerging as major concern to potato growers (Tiwari *et al*., 2020b, 2020a). These storage rots not only infest the tubers during storage and cause direct loss but also appear in fields in the form of wilts and stem rots that leads to indirect loss to the crop. These blemishes and lesion were earlier considered as minor damage but with the rapid advancement of processing industries the demand of blemish free potato tubers has increased manifolds. Moreover, the infection on tubers not only cause quantitative damage but qualitative loss in term of disturbed nutritional profile of tuber has also been reported (Kumar *et al*., 2020a, 2021a; Lal *et al*., 2020d, 2021c, 2021b; Singh *et al*., 2020; Raigond *et al*., 2021).

Among the skin blemish disease, the potato silver scurf caused by fungus *Helminthosporium solani* is a rapidly gaining importance as major challenge to potato cultivation and storage system. The disease is of worldwide economic importance due to its appearance in field at harvesting and during storage. The incidence of this disease may reach up to 80% as previously reported (Cunha and Rizzo, 2004). A survey-based study by (Nærstad *et al*., 2012) reported the incidence of silver scurf in 93% of potato samples comprised of 247 potato lots for skin blemishes in different regions of Norway. The silvery lesions covered 11-13% of the tuber area which was highest among all the skin blemish diseases. Previously, Bradshaw *et al*. (2002) reported 98% incidence of silver scurf and cited it most severe among skin blemishes of potato in cold stores of England and Wales. Likewise, in USA 75% of asymptomatic tubers were reported to possess *H. solani* infection in ten leading potato cultivars (Mattupalli *et al*., 2013). These observation across the areas of potato cultivation increases greater concern that the visual evaluation of tubers is not adequate to conclude that the tuber is free from silver scurf. The processing purpose potatoes face more challenges due to the infection as these silvery lesions enhance the permeability of periderm of tubers that cause weight loss and shrinkage of tubers (Raigond *et al*., 2020; Tiwari *et al*., 2020c; Kumar *et al*., 2021b; Lal *et al*., 2021b). The coloured potato cultivars lose the pigmentation due to heavy infection and the market value of fresh produce is severely hampered (Jellis, 1975). The disturbed processing quality of tubers can be estimated by the report that chips made from severely infected tubers comprise of blackish burnt appearance (Errampalli *et al*., 2001). The emergence of thiabendazole resistant strains from potato growing regions areas of the world has created more awareness in the exploration of suitable management practices for this disease (Hide *et al*., 1992; Merida & Loria 1990; Holley & Kawchuk 1996; Kutuzova *et al*., 2017; Kutuzova *et al*., 2017).

The present study mainly establishes the aetiology of this disease through morphological and molecular identification techniques. The isolation of pathogen from the tuber periderm is a difficult due to slow growing nature of the fungi. But the initial identification and spore visualization was performed with the help of symptomatic tubers via incubation at an optimum temperature range in a moist cabinet. Moreover, after the initiation of spores the pure culture was obtained through subculturing on suitable media. Further, the

mycelial boll formation and DNA extraction was performed, and fungus was molecularly identified using universal ITS primers. The pathogen culture and developed spore inundation technique will assist in *in vivo* identification of the fungus and development of suitable management strategies.

MATERIAL AND METHODS

Pathogen isolation and pure culture

During the year 2019, symptomatic potato tubers were obtained from commercial sheds in Madhya Pradesh. Approximately, 60% area of the tuber surface was covered with silvery lesions. The pathogen isolations were made by washing infected tubers with distilled water and tissue fragments were excised from lesion margins, disinfested in 5% NaOCl for 2 minutes, dried on sterile paper towel and placed on either clarified carrot juice agar media (Himedia) amended with 20 ml/L of 0.25% streptomycin sulfate, or potato dextrose agar (PDA, Himedia) amended with 0.25% streptomycin sulfate. The plates were incubated at 25° C in the dark and examined every other day (Cullen *et al.*, 2001). As soon as the mycelia growth was evident from the fragments the hyphal tip was transferred to a new plate with similar conditions. These fresh plates were also incubated in dark at 25° C and periodic observations were made for the fungus growth.

The standardization of spore inundation

The on-tuber spore proliferation was standardized to select a suitable temperature. The infected tubers were washed with running tap water, surface sterilized with 1% NaOCl for 2 min followed by drying. Three different temperatures (12, 18, 24°C) were selected to observe the proliferation of spore from the diseased lesions. The diseased tubers were kept in a dark moist cabinet at respective temperatures to maintain the relative humidity (>90%) for 21 days. Using a stereoscopic microscope, the progressive emergence of fungal assemblies on lesions were periodically observed. This technique was also used for pure culture isolation directly from the developed fungal growth on lesions. A small hyphal growth was excised using a sterile blade and placed on antibiotic amended half strength PDA. The plates were agitated for spore dispersion in the media and incubated at 25° C in dark for 4 days. Again, with the help of stereoscopic microscope the geminated single spore was transferred to fresh carrot juice agar media plates and incubated for 3 weeks. Symptoms were visually assessed and the range of sporulating lesions per tuber recorded. Using the previously described isolation approach, re-isolations were performed on every treatment replica (Rodriguez *et al.*, 1995). Three replications were maintained at each storage temperature. The statistical analysis was performed using GraphPad software.

Morphological and molecular identification

The colonies developed after pure culture were assessed for colony morphology and colour, the morphology of conidia and conidiophores. The mycelial plugs were observed using a dissecting microscope for conidiophore structure and a compound microscope for conidia. Isolates were confirmed as *H. solani* based on the previously described keys (Olivier and Loria, 1998). To observe the proper spore attachments with the conidiophore the double adhesive tape technique was utilized. Once the morphology was established the mycelial plug was inoculated to Potato dextrose broth and kept at 200 rpm for 21 days. The developed mycelia mat was strained in laminar cabinet and the DNA extraction was performed as previously

described by the method which includes phenol chloroform extraction (Mattupalli *et al*., 2013). The concentration of DNA was checked on nanodrop and it was stored at -20°C for further experimentation. Universal primers targeting internal transcriber spacer (ITS) region for the detection of *H. solani* were used. The primers ITS1, 2 amplified a fragment of 550-bp for detection of *H. solani* in through mycelium (Pérez *et al*., 2011). For ITS, initial denaturation was performed at 95°C for 3 min followed by 40 amplification cycles of 95°C for 45 s, 68°C for 45 s, and 72°C for 90 s. After 40 cycles, amplicons were incubated for another 8 min at 72°C for a final extension and a 10°C soak. To estimate the concentration and quality of PCR, 1 µL of amplicon from the PCR reaction was run on a 1.5% agarose gel with 0.5 µg of ethidium bromide ml–1 and 0.5 × Tris borate EDTA buffer for 45 min. DNA bands were visualized on a UV trans-illuminator. PCR amplicons were sequenced using the BigDye Terminator V2.0 ready reaction Kit (Applied Biosystems, CA, USA) and 0.5 × reactions.

RESULTS AND DISCUSSION

Pathogen isolation pure culture and symptomatology

The characteristic symptoms on the infected tubers were observed as blemishes or grey lesions on the periderm of tubers that appeared silvery when the tubers were moistened and incubated (Figure 1a, b). Discoloration of the lesions on periderm subsequently observed due to suberin deposition and cell desiccation. The silvery lesions were observed nothing but erumpent mass of conidia and conidiophores. The dispersed lesions later merge to develop into larger shiny silvery patches having a whorl of conidiophores bearing conidia in a typical basipetal fashion observed under a stereomicroscope. The pure culture plate of fungus has been provided in Figure 1 (c, d). Initially, the fungus appeared as hyaline mycelia which turned blackish brown on artificial media over the period of one month. The growth of mycelia was extremely slow, and it took more than three weeks to cover the petri plate (90mm diameter).

Spore proliferation on infected tubers

Three different temperatures were chosen to liberate the spores from the silvery lesions under 90-95% relative humidity conditions. The periodic observations revealed that maximum spore inundation occurred at 24°C as compared with 12°C or 18°C (Figure 2). At 12°C there were minute black dots which took 15 days in initiation and spore liberation was extremely low when counted using haemocytometer. The spore count was observed as 1.2×10^2 at the specified temperature. However, at 18°C the minute black dots appeared within 10 days and the hyphal tip initiation and spore mass was evident on the lesions after 20 days of incubation. The spore count was also elevated as it was observed up to 3.2×10^4.



Figure 1 a, b: the typical symptoms of potato silver scurf showing co-leased lesions and spore proliferations on tubers; c, d: colony growth of Helminthosporium solani.

Interestingly, at 24°C the initial of black blemishes occurred within a week and an erumpent mass of conidia and conidiophores were observed after two weeks on incubation. There was an extensive sporulation near the crevice and other depressions nearby stolon. The spore count had reached 2×10^5. The tuber eyes were also heavily colonized from the spore mass. These observations emphasize that the processing purpose tubers which are kept at 12°C are relatively safer from this disease as minor infection may not cause severe damage due to lack of sporulation. However, the tubers stored above that temperature and moreover the frequent fluctuation in the cold store that leads to build-up of heat may initiate the infection in other healthy tubers from the infected ones. Previous reports also highlight that table purpose potato stored at 4°C were less vulnerable than processing potatoes. Previously it was also reported that the conidial count on infected tuber surface was higher at 10-18°C as compared to 4°C (Rodriguez *et al.*, 1995). It was also recorded that the asymptomatic tubers developed scattered sporulation and shiny lesions after four weeks of incubation at 15 °C temperature and 90% relative humidity.

Morphological and molecular identification

The conidiophores were septate and had thin apex and broader base on which melanized conidia were developed in basipetal succession. The conidia possessed two to eight pseudosepta which arise in whorls on conidiophores (Figure 3 b, c). Conidiophores displayed the Christmas tree-like appearance, with the first conidium on the terminal position and 7 to 12 conidia arranged in a basipetal succession on the conidiophore (Figure 3c). The conidia were club shaped, cylindrical, melanized, 3–8 celled (Figure 3a). The size varied from 12.0 to 57.0 µm in length to 4.0 to 9 µm in width. Similar morphological features were depicted earlier and based on the characteristic appearance of conidiophores and conidial dimensions the fungus was identified as *H. solani* (Barnett and Hunter 1998). Similar pattern of conidiation, shape and size of conidia and pigmentation were also previously reported from semi-arid U.S. Pacific Northwest and western Washington (Inglis *et al.*, 2019; Miller *et al.*, 2015). The pathogenicity was proved on cultivars 'Kufri Pukhraj'.

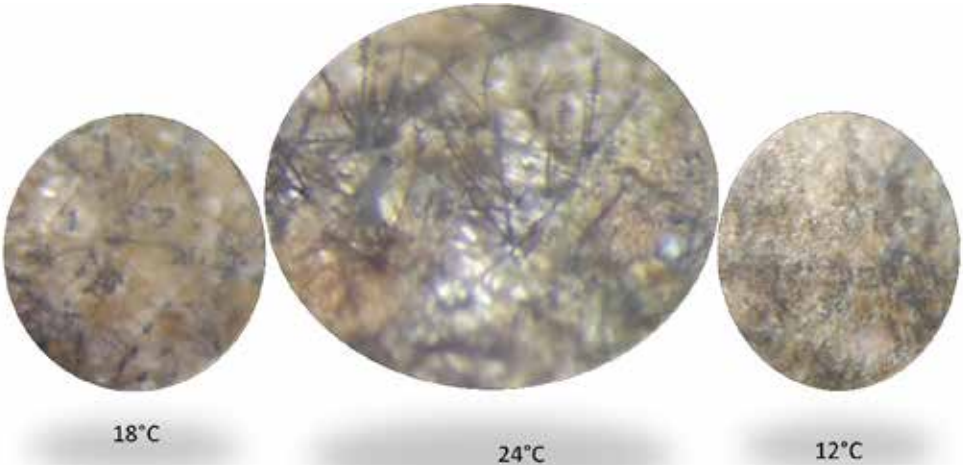


Figure 2: The pattern of sporulation at different temperature regimes

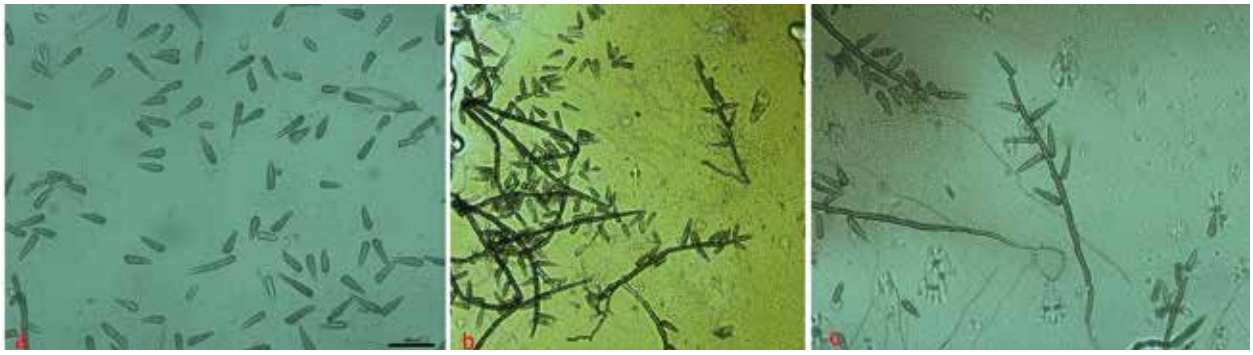


Figure 3: The morphological features of H. solani observed under compound microscope, a: solitary melanized conidia; b: the conidiophore bearing conidia at 20X; c: conidiophore and conidia at 40X

The morphological identification was further supplemented with molecular identification using ITS based PCR primers. The primer pair ITS1, 2, directed the amplification of a 550-bp target sequence in genomic DNA from the pure culture of fungus. The amplified bands were excised from the gel and purified GeneJET TM Gel Extraction Kit (Thermo Fisher Scientific). The products were sequenced using forward and backward primer (Eurofins Genomics India Pvt. Ltd). BLASTn analysis of the ITS sequence (Accession no. OK035227) showed more than 99% similarity (e-value: 0.0) to *H. solani* isolate CBS 365 (KY984341.1) and other isolates across the world. The phylogenetic tree was also constructed to analyse the evolutionary relationship (Figure 4). Previously the causal agent of silver scurf has been reported to be *H. solani* by using ITS based primers (Errampalli *et al.,* 2001). Mattupalli et al. 2013 utilized similar set of primers to identify the fungus from the asymptomatic organic potatoes.

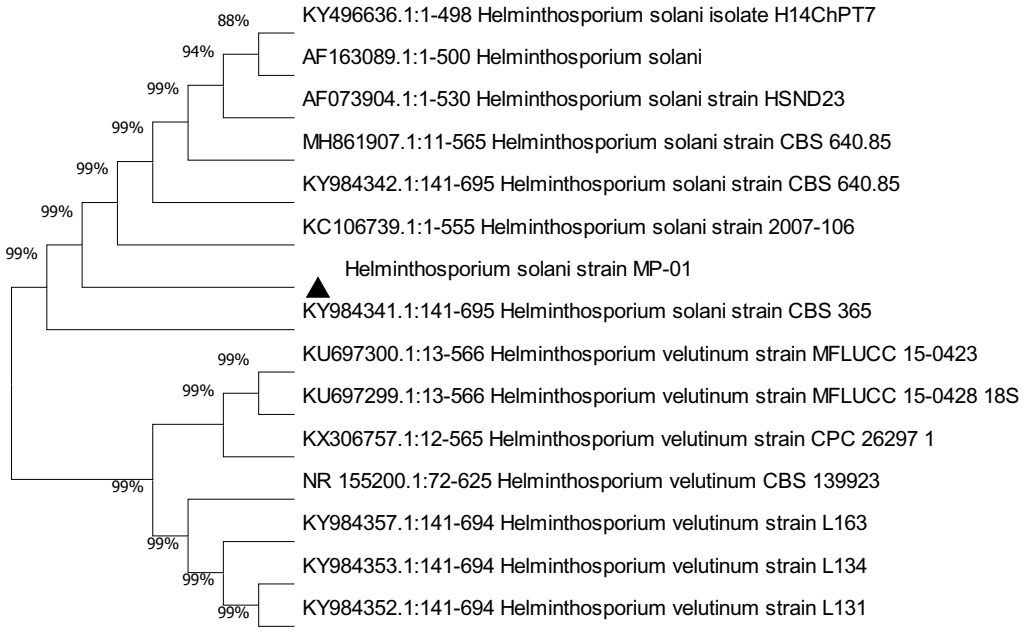


Figure 4: The phylogenetic tree of the isolate MP-01 constructed using neighbour-joining method using 1000 bootstrap value.

Subsequently the same group of researchers sequenced the whole genome of *H. solani* in the year 2014.

CONCLUSION

The potato silver scurf is a skin blemish storage disorder of potato having a worldwide concern. The diseased samples were obtained from a cold store and depicted typical silvery lesions scattered on the tubers. Initial observation and incubation gave an indication of *H. solani* infection. The spore proliferation was evaluated at a range of temperature, and it was observed that 24°C is the best sporulation temperature that produces the huge conidia mass in less time interval. The isolation and pure culture of pathogen was subjected to morpho-molecular identification and the aetiology of the disease was established. This information will assist in studying the epidemiological aspects of this disease coupled with designing of suitable management strategy.

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