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Morphological, cultural and molecular characterization of *Lasiodiplodia parva* causing leaf blight disease, firstly reported from India, on *Vanda* sp.

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Vanda (*Vanda* sp.), the most popular tropical orchid around the world, was attacked by blight disease that produced pale straw-coloured lesions throughout the leaf blade, coalesced, surrounded by a black margin, made the major portion of the leaf withered. Numerous, minute, dot-like, solitary, black, pycnidia (360.43 - 395.63 (av. 378.03) µm x 299.89 - 398.88 (av. 352.58µm) were produced. The immature single celled conidia obtained from culture had dimension of 14.87 - 20.61 (av. 17.98) µm and 8.08 - 11.0 (av. 9.42) ìm, length-breadth ratio and surface area of 1.54 - 2.36 (av 1.92) and 100.52 - 168.27 (av. 135.67) µm^2 respectively while each matured,chocolate brown, single-walled, ovoid, often bi-guttulated and bi celled conidia with a slight longitudinal stripe had dimension of 11.7 - 20.8 (av. 18.09) µm and 7.72 - 12.31 (av. 10.15) µm, length - breadth ratios of 1.13 - 2.15 (avg. 1.79) and surface area of 112.59 - 189.78 (av. 144.89) µm^2. The pathogenicity of the disease was established with purified culture. The fungus covered 9 cm diameter of Petri plate by 3 days on PDA, produced dry mycelial biomass of 0.310 - 0.365 g (av. 0.337 g) by 7 days on PDB medium. The production of pycnidia and spores production was possible to initiate 12 -15 and 20 -25 days after inoculation on PDA medium with dextrose @ 3.3g/ L. The causal fungus was identified as *Lasiodioplodia parva* by morphological and molecular characterization via amplification of the NS1/NS4 region of 18s rRNA. GenBank and accession number(PQ759907) and the type culture accession number (NAIMCC-F-04619) were obtained. This is the first report of *Lasiodiplodia parva* causing leaf blight on *Vanda* spp. from West Bengal and India as well.

Keywords : Biomass, *Lasiodiplodia parva*, leaf blight, radial growth, *Vanda*

INTRODUCTION

Vanda, a tropical orchid, has often been regarded as the '*prima donna*' among ornamental orchids in South and South-East Asia. It is mostly eulogized for its appealing, lucrative, myriad-shaped, multi-coloured,long-lasting and exquisite nature constituting an order of royalty in the world of ornamental plants (Zhang *et al*. 2018). Besides the aesthetic appeals,*Vanda* species are also important constituent of folk medicine, mainly in India, Nepal and China.

Due to complete tropical climatic requirement, ease of growing, high degree of cross-compatibility for the creation of new hybrid genera and suitability for mass propagation through *in vitro* culture, these orchids have gained immense significance in the global floral market over the last 20 years. But the production of this orchid is not keeping pace with the increasing demand due to potential biotic threats caused by the attack of 8 fungal,1bacterial and 5 viral diseases worldwide, out of which 4 fungal, 1 bacterial and 4 viral diseases are known to exist in India (Jain *et al.* 2021), but none of the diseases have been reported from West Bengal except 2 viral diseases (Pant *et al*. 2020). A severe leaf blight symptom, started to appear in late March to April, was observed at the study location and also from a number of commercial nurseries in the district during 2023-24. In the present study, attempts have been made to characterise the causal fungus, associated with leaf blight symptoms, by morphological, cultural and molecular means.

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MATERIALS AND METHODS

Vanda leaves showing typical blight disease symptoms were collected from experimental field of All India Co-ordinated Research Project (ICAR) Floriculture (Horticultural Research Farm) at Mondouri (22.56° N latitude and 88.32°E longitude), Bidhan Chandra Krishi Viswavidyalaya, Nadia, West Bengal, India. Infected portions were cut into small pieces, surface sterilized with 1% sodium hypochlorite solution for 45 seconds, rinsed serially 5-6 times with sterile distilled water and made blotted dry with sterilized blotting paper. Then, the pycnidia were picked from these leaf bits with the aid of a fine sterilized needle under stereo binocular microscope in the laminar air flow chamber. The stereo-binocular microscope was surface sterilized before use by wiping with 99% ethanol. Three pycnidia were picked up and transferred at three different places onto the solidified water agar medium in sterilized Petri plates, incubated in BOD at 28 ± 1°C and observed periodically for 1.5 – 2.0 cm growth of fungal mycelia.The bits of fungal growth from the isolation plates were transferred to potato dextrose agar (PDA) slants, incubated in BOD for 3-4 days. The fungal culture was maintained in PDA medium with subsequent subculturing. For determining the biomass production capacity, potato dextrose broth of 40 ml in 250 ml conical flask was inoculated with 8 mm diameter mycelial disc, incubated at 28 ± 1 ^{O}C for 7days. Mycelial mat was harvested and oven dried at 80^{O}C for 96 hrs to constant weight. Detailed morpho-metrical characterization of the fungal spores obtained from both the host tissue and culture media was performed using Carl Zeiss Axioscope microscope and Axiovision Rel 4.8.2. software. Length (L) and breadth (B) of conidia,L/B ratio and surface areas were noted from 30 randomly (n=30) selected conidia.The structure of pycnidia on the host tissue was studied with the aid of Zeiss stereo binocular and pycnidial dimensions from both culture and host tissues were also recorded. To establish the pathogenicity, a few healthy leaves were selected, washed thoroughly with sterile distilled water, made blotted dry and inoculated with 5mm disc of 48 hours' old active fungal culture. The inoculated leaves were kept in moist-chambered plastic trays, wrapped with transparent cellophane paper. For determining the molecular identity, the genomic DNA was extracted from mycelial mat using a modified CTAB (Cetyl trimethyl-ammonium bromide) method (White *et al*., 1990).The amplification of NS1/NS4 region of 18s rRNA was made using the primersNS1 **(**5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCTÒTAAG-3'). The gene sequence obtained was used to perform BLAST with the nucleotide database of NCBI GenBank. Based on the maximum identity score, first ten sequences were selected and aligned using multiple alignment software program Clustal W. The phylogenetic tree was constructed based on nucleotide sequences using MEGA 10 software considering 11 accession numbers *viz*. PQ759907 (isolated fungus), MN493107.1 (*Lasiodiplodia parva* isolate guava), KC442314.1 (*L. theobromae*), GQ469914.1 (*L. parva* strain CMW28309), GQ469913.1 (*L. parva* strain CMW28292), FJ215707.1(*Macrovalsaria megalospora* isolate 178150), PQ119511.1(*L. theobromae* isolate TL1), PQ119513.1 (*L. theobromae* isolate XZ1), NG_062745.1 (*L. theobromae* CBS 164.96), KC509580.1 (*Botryosphaeria laricina* strain JAS6 5.8S), PQ119514.1(*L. theobromae* isolate XZ2) to confirm the identity of the pathogen.

RESULTS AND DISCUSSION

Pale straw-coloured lesions were found throughout the leaf blade, starting from the base towards the tip of the leaves. As the infection progressed, several lesions coalesced, surrounded by a black margin, making the major portion of the leaf withered **(**Fig. 1a). Numerous, minute, dot-like, solitary, black, pycnidia of the fungus were produced at the collar region (mostly on the lower leaf surface), which was the typical diagnostic symptom of the disease (Fig 1b).The fungus was attempted to isolate using pycnidia. Its colony appeared within 48 hours after inoculation from all the pycnidia placed on the isolation plate (Fig 1c); mycelial bits were picked up from the colony margin and finally its purified culture was obtained. With a view to establish the pathogenicity, the healthy vanda leaves when inoculated with 48 hours old active pure culture, the symptoms of the disease were started to develop after 48 hours of inoculation. Initially, the

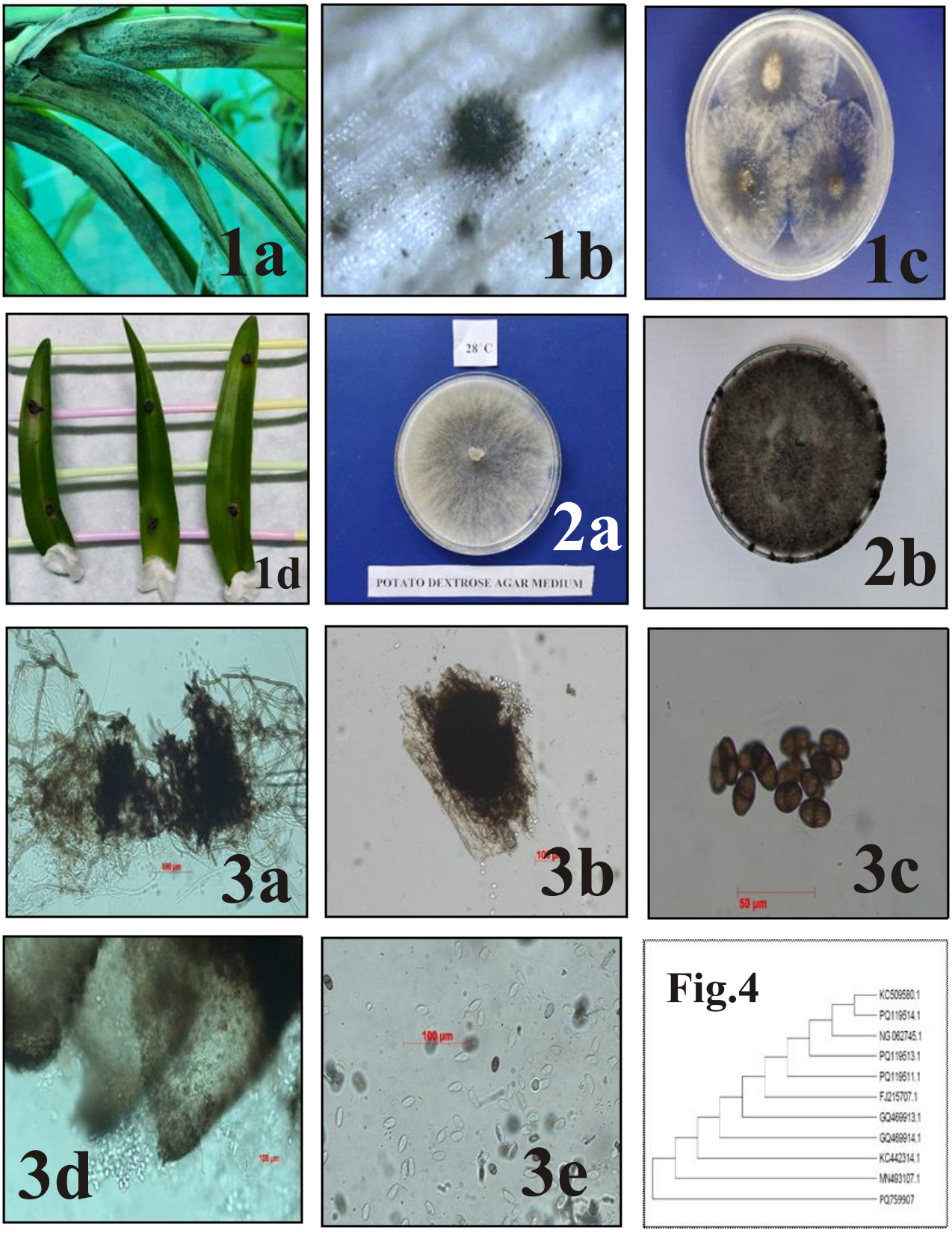

Fig.1a, 1b, 1c & 1d: Leaf blight symptom on *Vanda* sp.; Pycnidia releasing spores on host surface seen under binocular**;** Isolation plate **&** Pathogenicity establishment **2a & 2b:** *L. parva* after 72hrs of incubation in PDA at 28°C **&**after 7 days of incubation **3a, 3b,3c,3d, 3e & 3f**: Initiation of bird nest like structure prior to pycnidia formation (20X); Pycnidia obtained from host (10X); Bi-celled conidia obtained from host surface (20X); Pycnidia formation in 1/6th PDA medium (20X); Both single and bi-celled conidia production in 1/6th PDA medium (20X)**4:** Phylogenetic tree constructed using MEGA10

zone surrounding of inoculation point developed as pale yellow coloured lesion, which eventually exhibited withered appearance after 2 days with a progression on almost the entire leaf blade (Fig 1d). The inoculated leaf produced the same symptoms as it produced in the field under natural incidence of disease. The same pathogen as the initial one was re-isolated from the inoculated diseased leaf. In this way, the pathogenicity was firmly established following the Koch's postulates. Relatively similar leaf blight symptoms resulting in complete withering and drying of leaves were recorded on other orchid genera like, fringed catasetum (*Catasetum fimbriatum* (C. Morren) Lindl.) (Lopez *et al*. 2009) and *Dendrobium* spp. (Alam *et al*. 2023) both infected by *Lasiodiplodia theobromae*.

The colony of isolated fungus on PDA medium exhibited uniform, fluffy, dense, slight white to greyish coloured mycelial growth with a depressed centre at the front side where as it was compact and light white to greyish colour which later on turned into dark olive green to black colour at the backside. The studies made by Dheepa *et al*. (2018); Logeswari *et al*. (2022) hinted the initial fluffy whitish mycelium growth of *Lasiodiplodia theobromae* isolated from mango and coconut respectively, gradually turned into grey colour on aging as noted by us. The mycelia of the fungus covered the entire 9 cm diameter of Petri plate within 72 hours after inoculation on PDA medium at 28°C (Fig. 2a). The colour of entire fungal colony appeared dark olivaceous green to dark grey colour on both sides at 7 days after inoculation (Fig.2b).Similar mycelial characteristics with typical colour conversion pattern of both sides, except the appearance of pink pigmentation, was also noted by Fabiola *et al*. (2022) after 5 days of inoculation while studying the aetiology of black blotch causing fungi *Lasiodiplodia theobromae* on 'Guaria morada' or easter orchid [*Guarianthe skinneri* (Bateman) Dressler & Higgins]. In the present study, an array of dark pink pigments was observed in few Petri plates incubated at 28°C in PDA medium at 15 days after inoculation. Such type of frequent chromogenesis of vast array of pink pigment was also observed by Alves *et al*. (2008) in *Lasiodiplodia parva* (cryptic species of *Lasiodiplodia theobromae* species complex) fungal colony when incubated at 35°C.

Even after 3 months of inoculation, no pycnidia were found to produce on PDA medium with normal quantity of dextrose 20 g/ litre at 28°C. But at the same temperature regime, when the concentration of dextrose in PDA medium was reduced to 1/6th amount (i.e. 200g potato, 3.3g dextrose, 20g agar, 150 mg Chloramphenicol for 1 L PDA) dark black coloured, hard, scattered or intermingled, irregular-shaped 'bird nest' like pycnidia appeared on the edges of the Petri plate just after 12-13 days after inoculation (Fig. 3a). The fungus under study produced dry mycelial biomass of 0.310 - 0.365 g (av. 0.337 g) on PDB medium at 7 days afterincubation. Saha *et al*. (2008) recorded the mean mycelial dry biomass of 0.225±0.88 mg of *Lasiodiplodia the obromae*, isolated from tea, on PDB medium at 10 days after inoculation. They also mentioned that their test fungus did not sporulate in PDB medium with normal quantity of dextrose which partly supported our finding about the non sporulating behaviour of our isolate even after 3 months of incubation. The dry biomass produced by our test fungus, *L. parva* at 7 days after inoculation was registered more than that of mycelial dry biomass production of *L. theobromae* recorded by them at 10 days after inoculation. Such difference in biomass production could be possible due to the inherent variation of among species (Gourisankar *et al*. 2016)

From the diseased samples, elliptical, dark brown conidia were obtained by rupturing the minute, black coloured, globose to sub globose, sub-epidermal pycnidia (Fig 3b).The dimension of pycnidia obtained from the host were found between 360.43-395.63 (av. 378.03 ±31.53) µm x 299.89-398.88 (av. 352.58 ± 37.69) µm. Each of the conidium had a prominent septation at the middle, breadth highest at the middle and both ends were rounded. The length and breadth of the conidia obtained from the infected sample were ranged from 16.49 -24.86 (av. 20.29 ± 1.97) ìm and 10.96 -13.89 (av. 12.55 ± 0.85) µm respectively (Fig. 3c). The length - breadth ratios of conidia derived were found to vary from 1.29 - 1.88 (av. 1.62 ± 0.16) µm .Though the conidia were formed in ample amount from the affected host surface, but in *in vitro* condition,the spore production seemed to be quite difficult. Pycnidia obtained from PDA medium with 1/6th dextroseon

10 – 12 days after inoculation were found to release abundant oval shaped, double-walled, hyaline, aseptate, single-celled conidia initially. On further ageing of 13–15 days, these pycnidia started to release a few matured, chocolate brown, single-walled, ovoid, often bi-guttulated and bi celled conidia with a slight longitudinal stripe. Simultaneous production of both single and bi-celled conidia was observed, though the prevalence of single celled immature conidia was more in 25 days old culture grown on PDA medium with 1/6thdextrose**.** The dimensions of pycnidia obtained from culture were measured 278.88-439.67 (av. 343.24 ± 55.39) ìmx 196.55-305.4 (av. 256.84 ± 34.92) ìm, whereas ostioles were measured as 16.45-21.77 (av. 19.13 ± 1.94) ìm(Fig. 3d, 3e). The length and breadth of single celled conidia from culture were found in the range of 14.87-20.61 (av. 17.98 ± 1.53) μm and 8.08-11.0 (av. 9.42 ± 0.83) μm, length-breadth ratio and surface area were found within the range of 1.54-2.36 (av 1.92 ±0.19) and 100.52-168.27 (av. 135.67 ±1.53) μm^2 respectively whereas the dimensions of bi-celled conidia were found 11.7-20.8 (av. 18.09±1.92) μmx 7.72 -12.31 (av. 10.15 ± 0.95) μm respectively. The length to breadth ratio for bi-celled conidia ranged from 1.13-2.15 (avg. 1.79 ± 0.23) and the surface areas of spores were measured 112.59-189.78 (av. 144.89 ± 20.66) μm^2.In a description of cryptic speciation of *Lasiodiplodia theobromae* species complex *Alves et al.,*(2008) created the genus *Lasiodiplodia parva,*based on molecular characteristics and by differentiating it from *L. theobromae* in terms of smaller conidial dimension, shape of conidia and frequent appearance of an array of pink pigment in fungal colony (chromogenesis) when incubated at 35°C. This particular species was also documented by them to produce conidiomata on poplar twigs and uniloculate, dark brown to black, pycnidia immersed in the host which became erumpent on maturity. Pycnidia were found to produce ovoid conidia with broadly rounded apex, truncate base, thick-walled, initially hyaline and aseptate conidia, which on ageingbecame dark brown, one- septate with longitudinal striate appearance and dimensions were measured as (15.5-)16-23.5(-24.5) × (10-)10.5-13(-14.5) μm. This description of conidia perfectly fits our findings in terms of typical colony characteristics, conidial dimension and structure of pycnidia in host tissue, assuming the isolated fungus as *Lasiodiplodia parva* and also justifies the differences with the type species of this genus, *Lasiodiplodia theobromae*.

For more confirmation, the isolated fungus was also characterised by molecular means. A single discrete PCR amplicon band of 1050 bp was observed when resolved on 0.1% agarose gel. BLASTn analysis with nucleotide sequences of 18srRNA obtained from our sample exhibited 99.90% homology with *Lasiodiplodia parva* (Accession no. MN493107.1). The obtained sequence of leaf blight causing fungus from *Vanda* was deposited to NCBI GenBank and received an accession number PQ759907. The phylogenetic tree was constructed based on nucleotide sequences using MEGA 10 software considering 11 accession numbers *viz.* PQ759907 (isolated fungus), MN493107.1 (*Lasiodiplodia parva* isolate guava), KC442314.1 (*L.theobromae*), GQ469914.1 (*L. parva* strain CMW28309), GQ469913.1 (*L. parva* strain CMW28292), FJ215707.1 (*Macrovalsaria megalospora* isolate 178150), PQ119511.1 (*L.theobromae* isolate TL1), PQ119513.1 (*L.theobromae*isolate XZ1), NG_062745.1 (*L.theobromae*CBS 164.96), KC509580.1 (*Botryosphaeria laricina* strain JAS6 5.8S), PQ119514.1(*L.theobromae* isolate XZ2) to confirm the identity of the pathogen **(Fig. 4)**. Our isolate was found closely related to *Lasiodiplodia parva* (Accession no. MN493107.1). Finally, our fungal culture was submitted to NAIMCC and an accession number was assigned (NAIMCC-F-04619). Based on morpho-molecular characterization, our isolated fungus fits the description of *Lasiodiplodia parva* Phillips, Alves and Crous (MycoBank: 510942) (Alves *et al*. 2008). This particular species has been regarded as a cryptic species of *Lasiodiplodia theobromae*, which is actually a 'species complex' consisted of several cryptic species due to its wide adaptability and host range. These cryptic species are often morphologically indistinguishable but genetically differentiable.

CONCLUSION

Though a few reports on *Lasiodiplodia theobromae* were documented to induce diseases in a few commercial orchids, detailed search on

literature revealed no previous record regarding the infection of *Lasiodiplodia parva* from *Vanda* sp. To the best of our knowledge, this is the first report of *Lasiodiplodia parva* causing leaf blight on *Vanda* spp. from West Bengal and India as well. Considering the severity of the diseases, this study was conducted and concluded with the identification of disease based on diagnostic symptoms, confirmed the identification of fungal pathogen by morpho-molecular means as well as firmly established the association of causal fungus *Lasiodiplodia parva* with the disease through pathogenicity.

DECLARATION

The authors declare no conflict of interest.

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