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Research Article

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Bioherbicidal potential of *Rumex crispus* infected with *Didymella rumicicola*

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ABSTRACT

A new foliar disease was observed on a perennial weed *Rumex crispus* which grows extensively in orchards, pastures, hay crops, lawns and home gardens in Kashmir valley. Repeated Isolation of the pathogen from the infected leaves of *R. crispus* yielded the fungus which on the basis of Cultural and morphological characteristics showed a resemblance to *Didymella* sp. Pathogenicity was confirmed by the detached leaf technique as well as inoculating whole plants in pots. Precise confirmation of the identity of the pathogen was done by sequencing the reference genes using ITS1 and ITS4 markers and sequenced data was subjected to BLAST which showed 99.80 per cent similarity with *D. rumicicola* which was previously only reported in New Zealand on *R. obtusifolius* prior to this study. Host range as well as bioherbicidal potential of this pathogen on hosts of five different families of cultivated crops along with *Rumex* plants revealed that no disease incidence was found on host plant species of other families of cultivated crops, whereas,100% disease incidence and 80% severity were observed on *R. crispus* at 15 and 25 days after inoculation respectively. Physiological studies showed the newly isolated pathogen *D. rumicicola* showed the best radial growth on Potato dextrose agar at pH 6.5 and at a temperature of 25°C.

KEYWORDS: *Didymella rumicicola*, *Rumex crispus*, Bio-herbicide, Temperature, pH, Growth media

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INTRODUCTION

Rumex crispus L., the curled dock is a tap rooted stationary perennial weed, belonging to the Polygonaceae family. It is considered as one of the most widely distributed non-cultivated plants in the flora of the world (Hughes, 1938) and the most troublesome weed in many countries under temperate zones of the world especially among organic farmers where it is found to be abundant (Alexandra, 2008). As a follower of man, it has spread to every continent and is known as a serious weed of agriculture in many countries. The species occurs in a wide range of habitats, particularly waste grounds, road sides, shingle beaches, disturbed areas, temporary grasslands, and arable land. It is found on almost all soil types, except for the most acidic ones (Cavers & Harper, 1964).

A new foliar disease was reported on the perennial weed *R. crispus* from the Kashmir valley in India. Symptoms were noticed as small purplish red or reddish-brown spots which are conspicuous on both sides of the leaf and are often present in large numbers. Coalescing spots often resulted blighting of large leaf areas followed by necrosis. The objective of this study was to identify the pathogen associated with the host and to ascertain its possible bio herbicidal potential. Since the pathogen is not previously studied, in-addition to the above studies, some basic studies like culture media preferences, pH and temperatures for the favourable growth of the pathogen *in-vitro* were also carried out.

MATERIALS AND METHODS

Sample Collection and Fungal Isolation

Rumex crispus leaves showing typical disease symptoms were collected from the fields of Kashmir valley, India is situated at 34-20 (northern latitude) and 74 -24' east longitude at an altitude of 1610 meters above mean sea level (AMSL) during March 2017 when the weed plants were in rosette stage. The diseased leaves which exhibited symptoms of the disease were brought to our lab and inspected with the help of a microscope. The pathogen was separated from the host lesions by adopting the tissue bit technique as was done by Loladze *et al.* (2005) and Park *et al.* (2008). Samples of the diseased leaves were cut (using sterile micro scissors) into small bits which were about

1 cm in length and comprised of a portion of diseased tissue, and a bit of healthy tissue. The pieces were put in a 0.1 per cent mercuric chloride solution for exactly 30 secs, after which they were rinsed 2-3 times with the help of sterilized and distilled water to remove any traces of mercuric chloride solution. The pieces were then sterilely moved to disinfected Petri plates which contained sterilized filter paper. Thereafter, the pieces were moved to potato dextrose agar plates and were incubated for a period of 5-10 days at a temperature of 24±2℃. The procedure for isolation was replicated 15 times to ascertain the constant association of the pathogen with the host *R. crispus*.

Pathogen Recognition

Traditional morphological and cultural traits were used to identify the fungal isolate (which was already obtained) upon repeated attempts of isolation. The data of anatomical characteristics such as mycelia, conidia and pycnidia were based on a minimum of 50 measurements of each structure (which were found using an electronic microscope). Molecular characterization was performed using ITS markers. DNA extraction from the pure culture of isolated fungus was done using the protocol given by Doyle and Doyle method (Doyle & Doyle, 1990). The ITS (also known as the Internal Transcribed spacer) region of the rDNA was amplified with the help of a PCR which used ITS 1 and ITS 4 primers using: 94°C for 4 minutes, 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds, and 72°C for 8 minutes with the help of a peqSTAR 2X Thermocycler. The amplified product was custom sequenced by Genei Banglore. The sequence of PCR amplified product obtained from Genei Banglore was subjected to the NCBI (National Centre of Biotechnology Information) BLAST database and precise identification of pure culture was established. The sequence was submitted to the gene bank and secured in favour of SKUAST-K.

Pathogenicity Test

The pathogenicity was first confirmed by the detached leaf technique in the laboratory where 15 healthy surface sterilized leaves were used. Each leaf of *R. crispus* was placed over sterile glass slides kept in 90 mm sterile Petri plates lined with sterile blotting paper soaked in sterile distilled water. The leaves were sprayed with a spore suspension of isolated pathogen having a spore load of 1x106. Using a glass house, the pathogenicity test regarding the isolated fungal pathogens (one isolate) was reperformed on *R. Crispus* to confirm so. Following Koch's postulates, the pathogenicity of isolated pathogens was confirmed. The plants were transplanted in pots using intact root soil and apparently healthy plants in May 2017 (before flowering) when the plants were in the elongation stage. Twelve days old cultures of isolated pathogens were grown on potato dextrose (agar medium). They were then sprayed with 5 millilitres of distilled water and the conidia were loosened; the suspension was then passed through a cheesecloth. Conidial concentrations were adjusted to about 1 x 106 conidia ml^{-1} with the help of a haemocytometer. With the help of a Glass atomizer, the isolated fungal pathogens (at a concentration of 1 x 106 conidia mL^{-1}) was used to inoculate *Rumex* plants; thereafter the *Rumex* plants were incubated for 72 hours in a moist chamber. With one plant in each pot, twelve pots were inoculated. To serve a control, 3 un-inoculated pots (with one plant in each pot) were kept under similar conditions. To see whether symptoms were developing after inoculation, observations were recorded at regular intervals. Leaves, which developed characteristic symptoms, were used to re-isolated pathogens. In order to authenticate the identity of the pathogen and satisfy Koch's postulates; the culture received was compared to the original culture.

Host Range

The isolated pathogen was inoculated on five different plant species, for two consecutive seasons 2017-18. The seedlings were grown in pots representing different families along the stage of inoculation (Table 1). The plant species grown in pots in the poly house were sprayed during morning hours with a spore suspension of 1×10^6 spores/mL.

The inoculated pots were kept in a poly house and were studied for days to the appearance of the first symptoms and disease severity was to be recorded using a 1-9 severity scale (adopted from Schoonhoven & Pastor-Corrales, 1987), where 1 = no symptoms, 3 = lesions on 05-10 per cent of the leaf area, 5 = lesions and sporulation on 20 per cent of the leaf area, 7 = lesions up to 60 per cent of the leaf area, and 9 = lesions, frequently associated with the early loss of leaves and plant, on 90 per cent of leaf area. Percentage disease severity was calculated using the formula

$$\text{Percentage disease severity} = \frac{\sum(n\times v)}{N\times G}\times 100$$

Where,
n = No. of infected plants observed
v = numerical grade value
N = total no of plants observed
G = highest grade

Table 1: The isolated pathogen was inoculated on six different plant species, for two consecutive seasons in the years 2017-18. Seedlings were grown in the polyhouse (in pots) and were inoculated during morning time with spore suspension of having 1×106 spores per mL

	Family	Plant species that were tested	Stage of inoculation
1	Cucurbitaceae	Cucumber (*Cucumissativus*)	15 days after transplanting
2	Solanaceae	Tomato (*Solanum lycopersicum*)	15 days after transplanting
3	Fabaceae	Beans (*Phaseolus vulgaris*) (pole type)	2nd trifoliate stage (22 days after planting)
4	Cruciferae	Knolkhol (*Brassica oleracea*)	15 days after transplanting
5	Gramineae	Maize (*Zea mays*)	Knee height (35 days after planting)
6	*Rumex crispus*	*Rumex crispus*	Before flowering

Growth Media

The isolated pathogen was cultured on five different culture media namely, Potato dextrose agar, Oatmeal agar, Corn meal agar, Czapek's dox agar medium and Malt extract agar in BOD incubator, five replications of each medium were taken for study. Observations recorded were radial growth of pathogen (mm) after five days of incubation.

pH

The isolated pathogen was cultured at five levels of pH viz., 5.5, 6.0, 6.5, 7.0 and 7.5 in a BOD incubator. The pH of the media was adjusted at different pH levels with the help of a pH meter; five replications of each pH level were taken for study. Observations recorded were radial growth of pathogen (mm) after five days of incubation.

Temperature

The Isolated pathogen was cultured at five different levels of temperature viz., 10°C, 15°C, 20°C, 25°C and 30°C *in vitro* in five BOD incubators to determine the best growth of causal organism, five replications of each temperature level were taken for study. Observations recorded were radial growth of pathogen (mm) after five days of incubation.

Statistical Analysis

The data collected during the present investigations were subjected to appropriate statistical analysis using OPSTAT software given by Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Morphological and Cultural Characterization

The growth of the colony of the fungus isolated from the diseased leaves appeared within 24-48 hrs of incubation. The shape of the fungus colony is circular and its colour is initially tomentose olive-white in colour later turning to olivaceous grey forming black pycnidial fruiting bodies. Mycelium is septate with the dimensions of length×width of 15 – 20 µm×4 - 5 µm. Conidia are ellipsoidal, smooth, hyaline and aseptate with dimensions of length× width of 6 -7 × 1.5-2.5 µm (Figures 1 & 2). The fungus was tentatively identified as a *Didymella* sp. using the recently described generic descriptions in the *Didymellaceae* (Chen *et al.*, 2017).

Pathogenicity

The *Didymella* sp. was obtained upon repeated attempts of isolation and its pathogenicity was first conducted on detached leaves; symptoms begin to appear after 2-4 days of inoculation in the detached leaf technique. Thereafter, the pathogenicity was also ascertained by inoculating whole plants grown in pots where similar observations were obtained after the incubation period

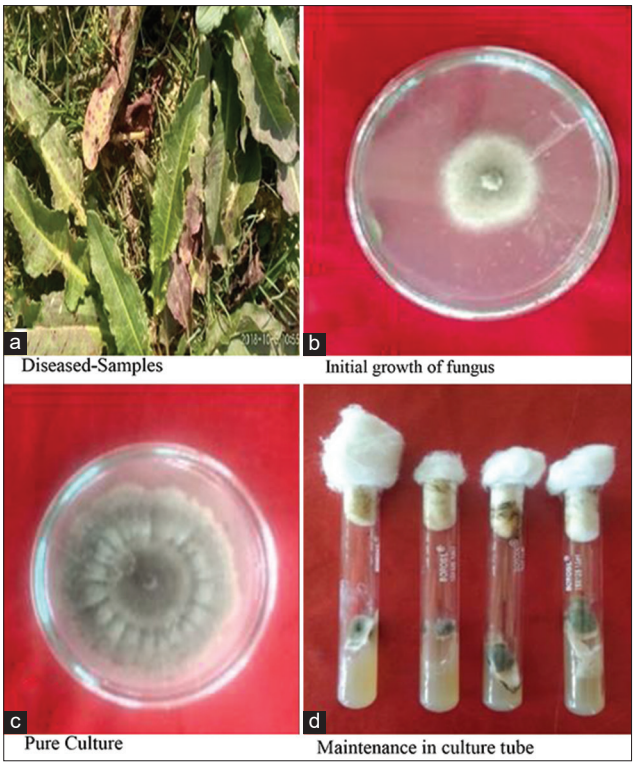

Figure 1: (a-d) Isolation of Didymella rumicicola from diseased leaves of Rumex crispus by using tissue bit technique. The growth of the colony of the fungus appeared within 24-48 hrs of incubation

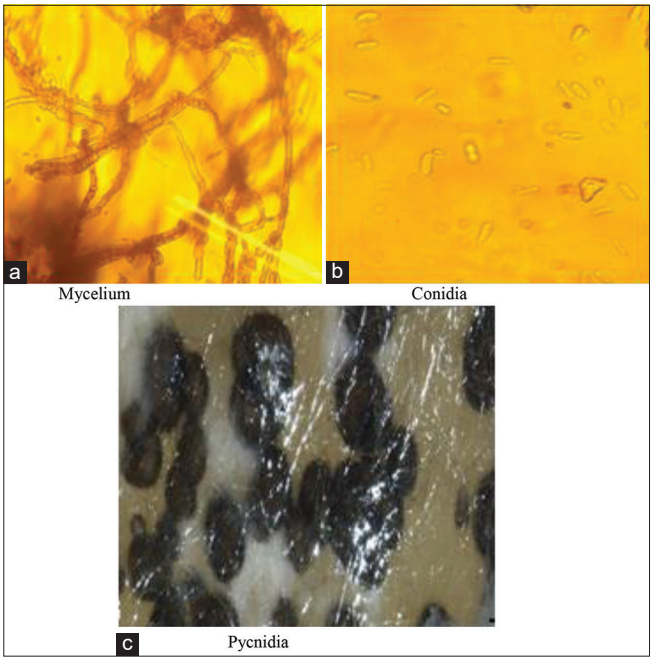

Figure 2: (a-c) The morphological data e.g. mycelia, pycnidia, conidia of Didymella rumicicola were based on at least 50 measurements of each structure under a light microscope (Olympus CX51, Japan). The shape of the fungus colony is circular, and its colour is initially tomentose olive-white in colour; later turning to olivaceous grey forming black pycnidial fruiting bodies. Mycelium is septate with the dimensions of length × width of 15 – 20 µm ×4 - 5 µm. Conidia are ellipsoidal, smooth, hyaline and aseptate with dimensions of length × width of 6 -7 × 1.5-2.5 µm

of 4-5 days no symptoms were observed in control treatments which were sprayed by only sterile distilled water.

Molecular Characterization

Upon sequencing of the amplified PCR product, a 570 base pairs sequence was obtained. Upon conducting BLAST, the sequence gave 99.80 per cent similarity with *Didymella rumicicola*, the sequence was submitted to the NCBI database under accession no. MK412085 and named SKU1.

Host Range and Bioherbicidal Potential

In our present studies, inoculation of *D. rumicicola* on the host plant species of five different families of cultivated crops viz. Gramineae (*Zea mays*), Fabaceae (*Phaseolus vulgaris*), Solanaceae (*Solanum lycopersicum*), Cruciferae, (*Brassica oleracea*) and family Cucurbitaceae (*Cucumis sativus*) grown in pots in the poly house have not produced any symptoms other than *Rumex sp*. On the other hand, this pathogen showed typical disease symptoms in 4-5 days when inoculated on leaves of *Rumex* plants. The mean disease incidence of 100% and severity of 57 % was observed within 15 days of inoculation and reached up to 80 per cent severity after 25 days of inoculation on *Rumex* plants (Table 2).

Growth Media

Upon growing on different culture media, it was observed that this pathogen showed the best growth on Potato dextrose agar (20.8 mm) followed by Oatmeal agar (16 mm), Malt extract agar (14.11 mm), Corn meal agar (13.2 mm) and Czapex'sdox agar (12.55 mm) after five days of incubation (Figure 3).

pH

At five different levels of pH tested in vitro *viz*., 5.5, 6.0, 6.5, 7.0 and 7.5, it was observed that *Didymella rumicicola* grows best at pH 6.5 (22.30 mm) followed by pH 7.0 (19.00 mm), pH 5.5 (18.5mm), pH 6(17.22mm) and least at pH 7.5 (16.30 mm) after five days of incubation (Figure 4).

Table 2: The table represents the bioherbicidal potential of The *Didymella rumicicola;* on hosts of five different families of cultivated crops, namely Gramineae (*Zea mays*), Fabaceae (*Phaseolus vulgaris*), Solanaceae (*Solanum lycopersicum*), Cruciferae, (*Brassica oleracea*) and family Cucurbitaceae (*Cucumis sativus*) along with Rumex plants revealed that no disease incidence was found on host plant species of other families of cultivated crops, whereas, 100% disease incidence and 80% severity were observed on *Rumex crispus* at 15 and 25 days after inoculation respectively.

No. of days	Percent (%) Disease Incidence	Percent (%) Leaf area infected
5	46.50	15
10	76.60	33
15	100	57
20	100	68
25	100	80

Temperature

At five different levels of temperature *viz*., 10, 15, 20, 25 and 30°C tested in vitro, *D. rumicicola* grows best at temperature 25°C giving a radial growth of (21.22 mm), followed by growth at temperature 20°C giving a radial growth of (19.77 mm) and the least growth of (9.9 mm) was recorded at temperature 30°C after five days of incubation (Figure 5).

Molecular sequencing of the tentatively identified fungus *Didymella* sp. based upon the generic descriptions given in the *Didymellaceae*, yielded a 570 bp sequence of amplified PCR product. This sequence when subjected to BLAST on the NCBI database showed 99.80 per cent similarity with *D. rumicicola* which was previously reported in New Zealand (Chen *et al*.,

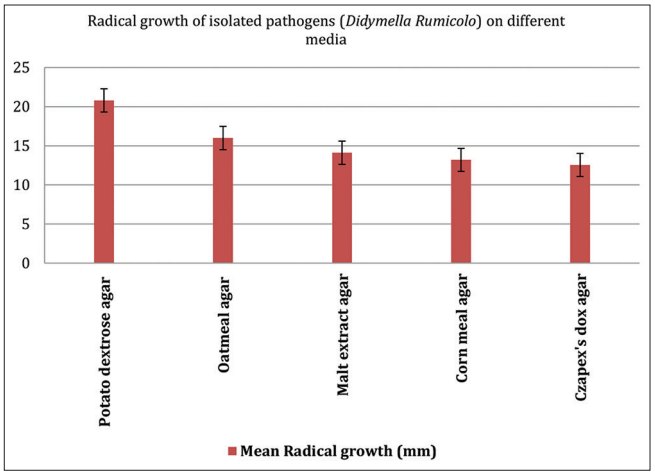

Figure 3: Upon growing on different culture media, it was observed that *Didymella rumicicola* showed best growth on Potato dextrose agar (20.8 mm) followed by Oatmeal agar (16 mm), Malt extract agar (14.11 mm), Corn meal agar (13.2 mm) and Czapex's dox agar (12.55 mm) after five days of incubation

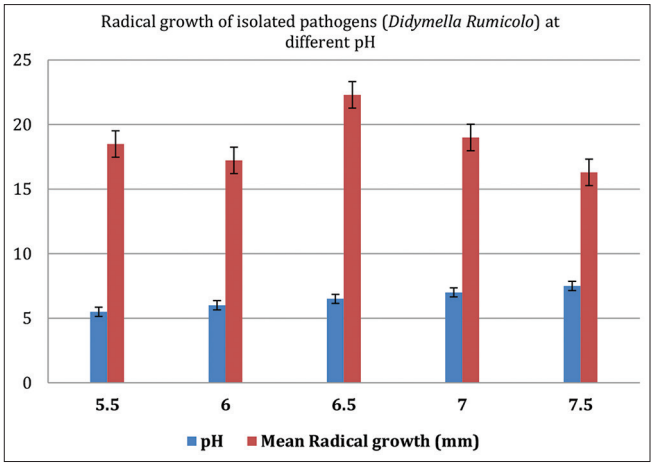

Figure 4: The isolated pathogen, *Didymella rumicicola* was cultured at five levels of pH viz., 5.5, 6.0, 6.5, 7.0 and 7.5 in BOD incubator. The pH of media was adjusted at different pH levels with the help of pH meter; five replications of each pH level were taken for study. Observations recorded were radial growth of pathogen (mm) after five days of incubation

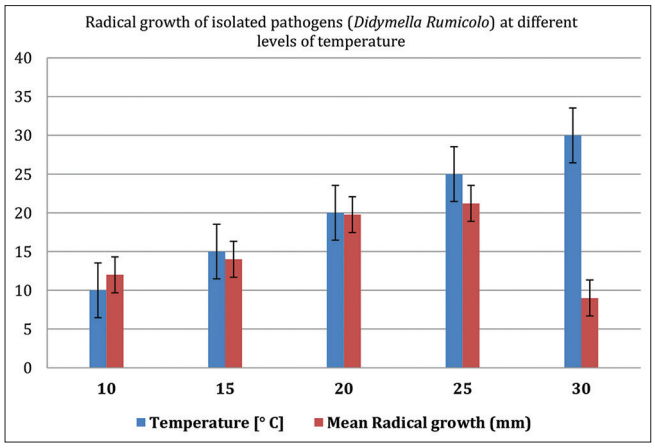

Figure 5: *Didymella rumicicola* was cultured at five different levels of temperature viz., 10°C, 15°C, 20°C, 25°C and 30°C in vitro in five BOD incubators to determine the best growth of causal organism, five replications of each temperature level were taken for study. Observations recorded were radial growth of pathogen (mm) after five days of incubation.

2017). After extensively consulting the literature about this pathogen, the isolated pathogen was not yet reported from Asia especially on the perennial weed species *R. crispus*. So, this is the first report of *D. rumicicola* from sub-continent India as well as from continent Asia.

Although different species of *Didymella* infecting hosts of different families were reported, in our present studies, inoculation of *D. rumicicola* on the host plant species of five different families of cultivated crops grown in pots in the poly house has not produced any symptoms other than *Rumex sp*., indicating the narrow host range of *D. rumicicola* and its use as bioherbicide targeting only the perennial weeds of *Rumex* sp. This study points towards the fact that *D. rumicicola* can be safely used to control *Rumex* sp. Opening opportunities for its secure use as bioherbicide against *Rumex* sp. However further testing on the large vast number of cultivated crop varieties needs further to be conducted. The Previous report of *D. rumicicola* causing leaf spots was also confined to *Rumex* sp. namely *R. obtusifolius* (Boerema *et al.*, 1980). The mean disease incidence of 100% recorded within five days and severity up to 80 per cent after 25 days of inoculation on *Rumex* plants clearly indicate the potential of *D. rumicicola* as bioherbicide against *Rumex* sp. Previous reports of rust fungus *Uromyces rumicis* and necrotrophic fungus *Ramularia rubella* were most studied as biological control agents against *Rumex* sp. Especially *R. crispus* and *R. obtusifolius*, where *U. rumicis* in the 1960s was considered as a potential bioherbicide against *R. crispus* in USA and *R. rubella* in Europe against these dock species (Inman, 1970; Hatcher *et al.*, 2008). Since there were no previous reports mentioning the use of *D. rumicicola* as a fungal bioherbicide against *Rumex* sp., the present study first time reports its possible bio-herbicidal potential which paves the way for opening new possibilities of research in order to commercially exploit this pathogen in *Rumex* weed management especially in organic farming systems of the world.

D. rumicicola when tested for its mycelial growth in vitro on various growth producing media at different levels of pH and temperatures found that maximum radial growth of mycelium was obtained on potato dextrose agar media followed by oat meal agar at pH 6.5 when incubated at 25°C temperature indicating that mass production and commercial formulations of the pathogen can be well carried at these optimal conditions in the laboratory. Previous studies on *Phoma rumicicola* (syn; *D. rumicicola*) growing very rapidly on oatmeal agar, malt-extract agar, and cherry agar have already been reported (Waller *et al.*, 1968). Maximum growth, fructification and spore germination of *D. bryoniae* causing blight in ridge gourd has been reported on potato dextrose agar and corn meal agar media when maintained at pH 7.0 and incubated at temperature 24±1°C (Bhat *et al.*, 2009). Although with minor deviations from previous reports, our present studies are in agreement with them apart from yielding some new findings.

CONCLUSIONS

The information in this study plays a vital role in understanding the etiology of the foliar disease on of *R. crispus* caused by pycnidial fungus *D. rumicicola* and also its bioherbicidal potential in checking the population of troublesome perennial weed *Rumex* sp. thereby increasing the scope of future prospects of research in possible commercializing the formulation of this pathogen to be used as a bioherbicide.

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