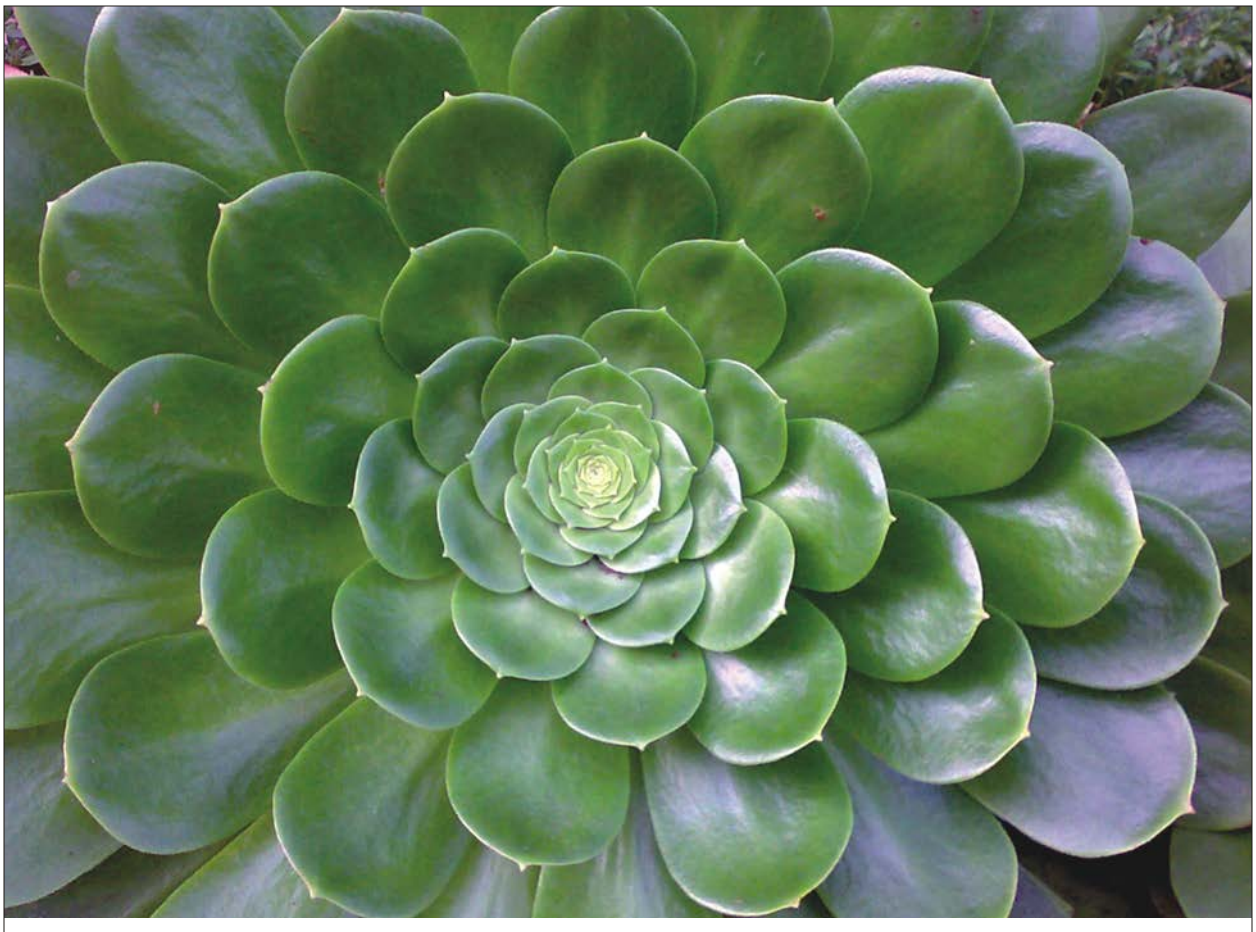

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Research Article
Neopestalotiopsis chrysea Causing Leaf Spot Disease of Strawberry Plants in Bangladesh

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Abstract

Background and Objective: Strawberry leaf spot diseases caused by fungi are the most common and economically significant concern, reducing strawberry productivity and quality. Current investigation attempts to find out the causal agent of certain leaf spot disease of strawberry, growth behaviour of the pathogen and its *in vitro* control strategies. **Materials and Methods:** A fungal pathogen causing leaf spot of the strawberry plant was isolated from a distinguish symptom and identified as *Neopestalotiopsis chrysea* (Maharachch. and K.D. Hyde) Maharachch., K.D. Hyde and Crous. Through classical taxonomy and molecular characterization based on sequencing the internal transcribed spacer region of rDNA of the fungus. The growth behaviour of the fungus was evaluated on six different solid fungal culture media, different light, temperatures and pH conditions. Dual culture and food poison techniques were applied to evaluate the efficacy of *Trichoderma* spp. and chemical fungicides against the fungus, respectively. **Results:** The optimum mycelial growth of the fungus was recorded on potato dextrose agar and potato sucrose agar media, at 20°C temperature, pH 7 and under complete dark conditions. *Trichoderma reesei* exhibited significant mycelial growth inhibition of the fungus. The maximum mycelial inhibition of the fungus was found in amistar top (100 ppm), followed by tilt 250 EC (500 ppm). **Conclusion:** To the best of our search, the leaf spot disease of strawberry caused by *Neopestalotiopsis chrysea* is a new record in Bangladesh.

Key words: Molecular identification, culture media, temperature, pH, fungicides

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Strawberry (*Fragaria× ananassa* Duch.) is a stoloniferous and perennial herb under the family Rosaceae. There are 500 commercial cultivars of strawberries worldwide and 71 counties are commercially cultivating strawberries[1]. The flowering of the strawberry plants is determined by critical day length and environmental factors. For maximum strawberry production, short photoperiod and lower day temperatures are key conditions[2]. In Bangladesh, the winter season is most suitable for strawberry cultivation[3]. Hence, Bangladesh Agricultural Research Institute and Bangladesh Agricultural Development Corporation have released a few strawberries varieties such as BARI strawberry-1, BARI strawberry-3 and BADC strawberry[1,4]. Fungi may cause disease on different parts of strawberry plants such as on leaves, fruits, crowns and roots. Fungal pathogen *Pestalotiopsis* species causing crown rot diseases on strawberries were reported in Vietnam[5], Bangladesh[6] and Spain[7]. Besides, *Pestalotiopsis longiseta* causing leaf spot disease of strawberry in Brazil[8] and *Neopestalotiopsis rosae* causing strawberry root and crown rot in Egypt[9] were also reported and known to be responsible for substantial economic loss of farmers. Molecular identification of fungi via sequencing of the internal transcribed (ITS) region of rDNA has become an essential part of fungal taxonomy research[10]. Therefore, the current study was undertaken to isolate and identify strawberry leaf pathogen using the molecular approach, to investigate the fungal biology (culture media, light, temperature and pH), to assess the efficacy of bio-control agents and synthetic fungicides against the isolated fungus.

MATERIALS AND METHODS

Study area: The field was prepared in the experimental sites of Botanical garden (N: 23°52'16", E: 90°15'56"), Jahangirnagar University, Savar, Dhaka, Bangladesh with the randomized complete block design. Five beds were prepared (100×280 cm) and twenty strawberry saplings were transplanted in two rows in each bed. The distance between the two plants was 40-50 cm. A 50 cm drain was prepared between two beds. Strawberry saplings were planted at the end of November, 2016 and harvested in March, 2017.

Isolation and identification: Diseased strawberry leaves with distinctive symptoms were collected from the experimental field and the tissue planting method was used to isolate fungal pathogens. Morphological identification was carried out based on the macroscopic study of the colony, microscopic study of mycelium and conidia.

Two universal primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) was used to amplify the target region of the fungus as according to Mallik *et al.*[11]. The PCR reaction was carried out as the protocol described by Sikder *et al.*[12]. The purified PCR products of approximately 650 bp were sequenced in first BASE Laboratories Sdn Bhd (Malaysia). The phylogenetic tree was generated using MEGA 6 software.

Effect of culture media and physical factors on the vegetative growth of the fungus: The mycelial growth of *N. chrysea* was measured using six distinct cultural media viz., Potato Dextrose Agar (PDA), Potato Sucrose Agar (PSA), Carrot Agar (CA), Honey Peptone Agar (HPA), Richard Agar (RA) and Hansen's Agar (HA) as the method mentioned by Sultana *et al.*[13]. The effect of temperatures (10, 15, 20, 25 and 30°C) on the vegetative growth of the fungus was evaluated as the methods described by Sultana *et al.*[13]. The effect of light (24 hrs light, 24 hrs dark and alternate cycle of 12 hrs light and 12 hrs dark) on the vegetative growth of the fungal pathogen was investigated as the method described by Sikder *et al.*[14]. The five different pH conditions (pH 5, pH 6, pH 7, pH 8 and pH 9) were also investigated to find out the effect of hydrogen ion concentrations on fungal growth[15]. Data on the mycelial growth was recorded at 7 days post-inoculation (dpi).

***In vitro* mycelial growth inhibition of the fungus:** The mycelial growth inhibition of *N. chrysea* was investigated using a dual culture approach with biological control agents *Trichoderma reesei*, *T. harzianum* and *T. asperellum*. A range of fungicides-Tilt 250 EC and Amistar Top 325 SC @ 100ppm, 250ppm, 500ppm, and Ridomil gold MZ 68 WP, @ 250ppm, 500 and 750 ppm were assessed to know the inhibitory activity against the isolated fungus using poison food technique[16]. As a control, PDA plates containing no fungicides were used. The mycelial growth inhibition percentage of the fungus was calculated at 7 dpi following standard formula:

$$I = \frac{C\text{-}T}{C} \times 100$$

Where,

I = Percentage of vegetative growth inhibition
C = Growth of mycelium in control plates
T = Growth of mycelium in treatment conditions

Statistical analysis: Data on the effects of fungal culture media, light, temperature, pH, antagonistic fungi and chemical fungicides on mycelial growth of the isolated fungus were found to be normal and analyzed using SPSS-20.

RESULTS AND DISCUSSION

Symptomatology and identification of the fungus: Leaf spots first appeared on the upper surface of the leaf as a circular, deep purple colour symptom. With the ageing of leaves, the spots became larger and the cores of the spots turned greyish to white and light brown. A definite reddish-purple to rusty brown border surrounded the spots in Fig. 1a. Middle-aged leaves were most susceptible to this disease.

The colony of the fungus was white in colour, cottony, circular and slightly fluffy on PDA media in Fig. 1b. Black oozing appeared on the colony. It had hyaline and septate mycelium. Conidial mass was black on PDA. Conidia were fusiform to ellipsoidal, four septate and had five cells. Apical and basal cells of conidia were hyaline in colour and the three median cells ranged from light brown to dark brown. Conidia had two to three apical appendages Fig. 1c. Based on colony appearance and conidial features, the fungus was identified as *Neopestalotiopsis* sp. Further molecular analysis was performed to identify the fungus at species rank.

The ITS sequence of this fungus was deposited in NCBI GenBank under the accession number: MH371470.1. The obtained sequences showed 99% similarity with *Neopestalotiopsis chrysea* (KU534877.1 and MT459336.1) of NCBI Genebank. The maximum likelihood tree was generated by combining all sequenced retrieved from NCBI Genebank in MEGA 6 software. Our studied fungus was clustered in the *N. chrysea* clade with 99% bootstrap support in Fig. 2.

Effect of fungal culture media, temperature, light and pH on the vegetative growth of the fungus: The effect of six different solid media (PDA, PSA, RA, CA, HPA and HA) on the mycelial growth of *N. chrysea* was evaluated under *in vitro* conditions in Fig. 3 and 4. There was a significant difference among the cultural media to support the growth of the fungus. The result revealed that the highest (90 mm) mycelial growth of *N. chrysea* was recorded on both PDA and PSA media, followed by 74.5 mm on RA medium, respectively, while the lowest (57.5 mm) mycelial growth was recorded on HPA medium at 7 dpi. Our study suggests that both PDA and PSA media were suitable for the mycelial growth of *N. chrysea*. Likewise, El-Gali[17] observed that PSA media was the best for the growth of *Pestalotiopsis* sp. Contrary, Fovo *et al.*[18] stated that the radial growth and conidia concentrations of *Pestalotiopsis microspora* were maximum on V8 juice agar compared to PDA. Besides, Bajo *et al.*[19] reported the highest growth rate of *Pestalotiopsis funerea* on the TAKAY medium compared to the other four different media since the TAKAY medium consists of many compounds and nutrients.

The effects of different light conditions-24 hrs light, 24 hrs dark and 12/12 hrs light-dark on mycelial growth of *N. chrysea* on PDA media was assessed in Fig. 5. The maximum (62 mm) mycelial growth of *N. chrysea* was measured under complete dark conditions, followed by 42.83 mm in alternate light and dark conditions while the minimum (30.33 mm) mycelial growth of the fungus was measured under continuous light conditions. Likewise, El-Gali[17] worked on the effect of ecological factors on the growth of *Pestalotiopsis* spp. (*Pestalotiopsis fici*, *P. guepinii* and *P. palmarum*), in which the best mycelial growth of *Pestalotiopsis* spp. was recorded under continuous dark state and growth rate was reduced under continuous light state. Besides, *P. microspora* grew well under the continuous dark conditions[20].

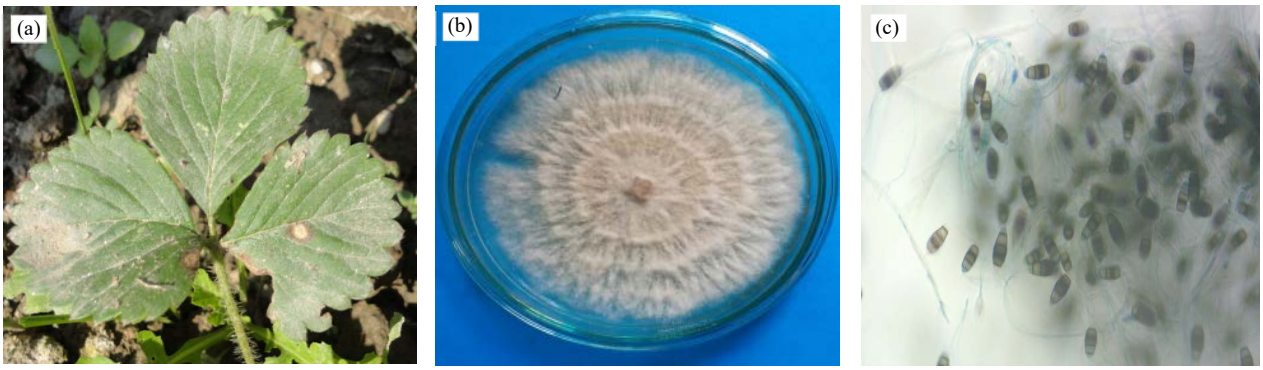

Fig. 1(a-c): Strawberry leafs (a) Symptoms of *N. chrysea* on strawberry leaf, (b) Vegetative growth of the fungal mycelium on PDA media and (c) Microscopic views of *N. chrysea* (400X)

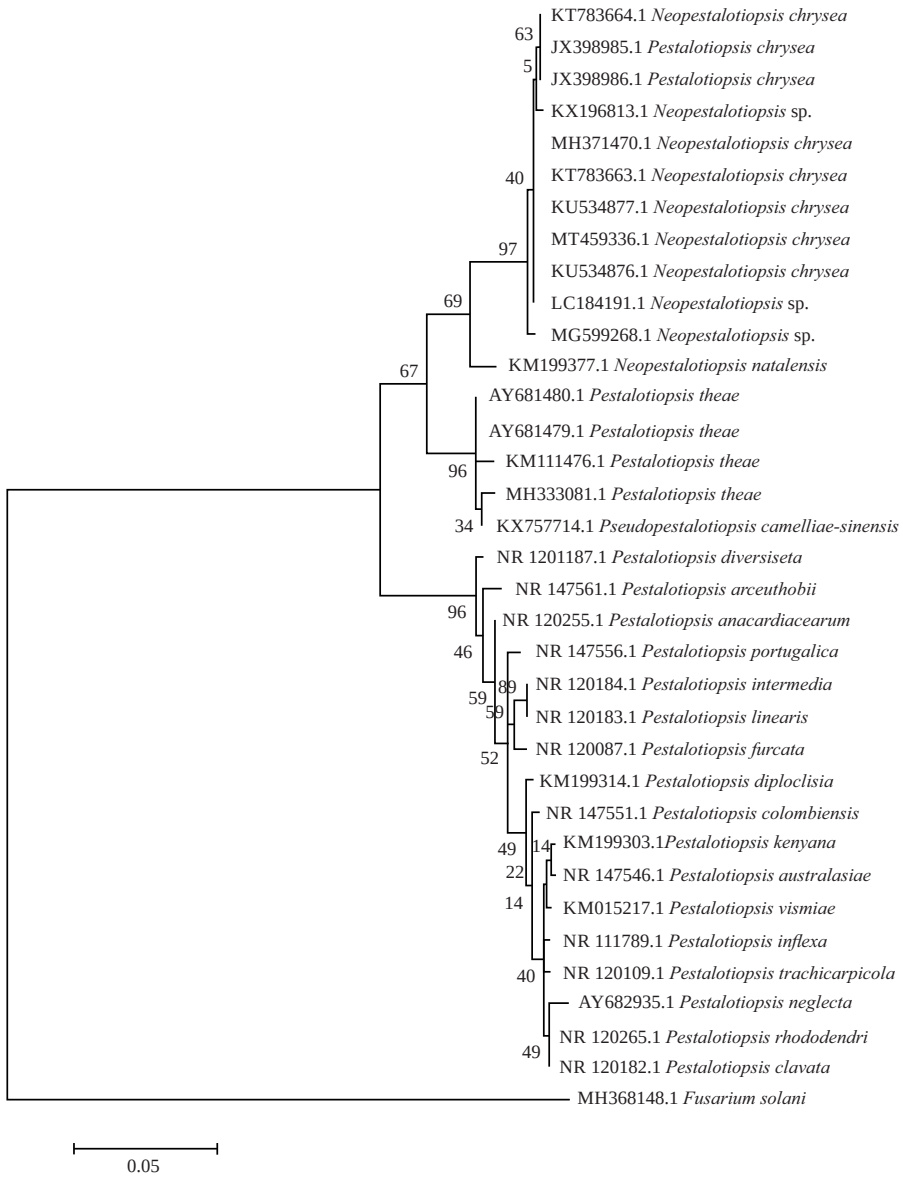

Fig. 2: Maximum likelihood tree of the studied organisms with bootstrap value (Replication = 1000)
NCBI accession numbers are mentioned before the genus name. Our organism is MH371470.1

An optimum temperature plays a regulatory role in the development and growth of fungi. Five temperature conditions evaluated on the mycelial growth of the fungus on PDA media under *in vitro* conditions in Fig. 6. Results revealed that the highest (72.5 mm) mycelial growth of *N. chrysea* was recorded at 20°C temperature, which was significantly different from other treatments and the lowest (13.67 mm) growth was obtained at 35°C temperature. Interestingly, there was a gradual decline of the mycelial growth of the fungus after 20°C temperature. Our results are supported by El-Gali[17], who found the maximum growth of *Pestalotiopsis* species (*Pestalotiopsis fici*, *P. guepinii* and *P. palmarum*) at 20-30°C temperatures conditions. Moreover, Fovo *et al.*[18] cited that the growth rate and conidia concentration of *Pestalotiopsis microspora* was increased with temperature and reached their peak growth at 23°C. Likewise, Keith *et al.*[21] reported that the optimum growth temperature of *P. microspora* may differ between 22 and 28°C depending on fungal isolates. Furthermore, it was reported that *Pestalotiopsis theae* did not grow at 35°C, probably due to the inactivation of necessary enzymes by higher temperature as a result, fungal metabolism was interrupted[22,23].

The variation in hydrogen ion concentration of fungal media correlates with the mycelial growth of almost all of the fungi. The suitable pH of culture media for the best growth

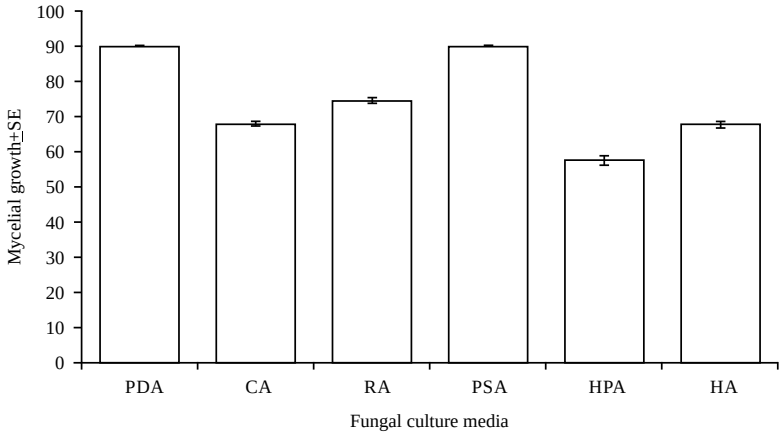

Fig. 3: Effect of fungal culture media on mycelial growth (mm) of *N. chrysea* at 7 dpi

Value represents Mean±Standard error of nine replications , PDA: Potato dextrose agar, CA: Carrot agar, PSA: Potato sucrose agar, RA: Richard agar, HPA: Honey peptone agar and HA: Honey agar

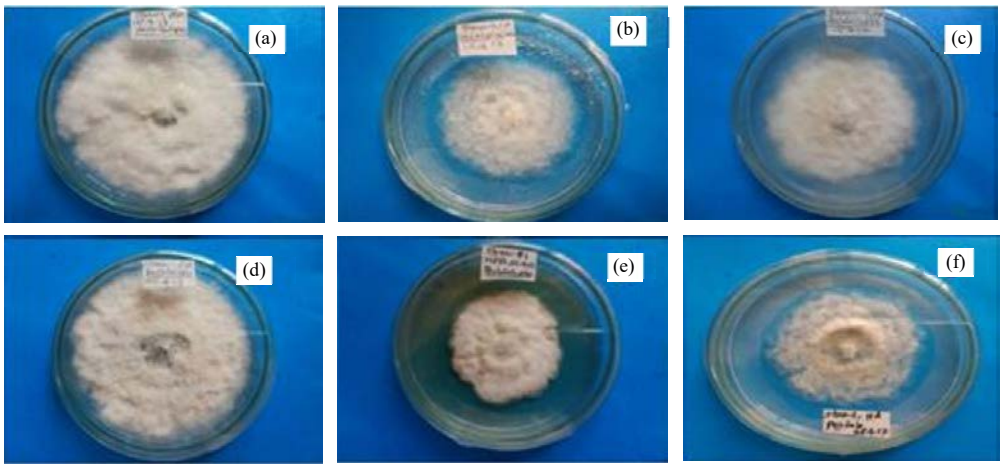

Fig. 4(a-f): Effect of culture media on vegetative growth of *N. chrysea* at 7 dpi, (a) Potato dextrose agar, (b) Carrot agar, (c) Richard's agar, (d) Potato sucrose agar, (e) Honey peptone agar and (f) Hansen's agar

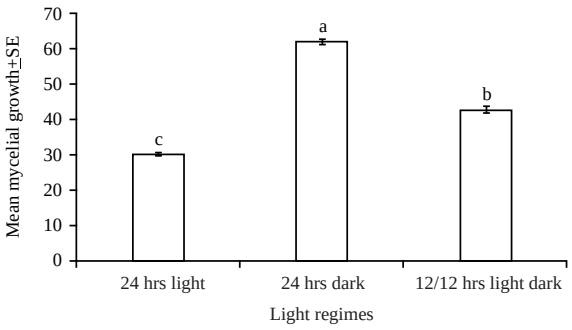

Fig. 5: Effect of light on mycelial growth (mm) of *N. chrysea* at 7 dpi

Value represents Mean±Standard error of nine replications, significant differences are presented as alphabet on the top of the bar and same letter do not differ significantly at 5% level of significance (DMRT)

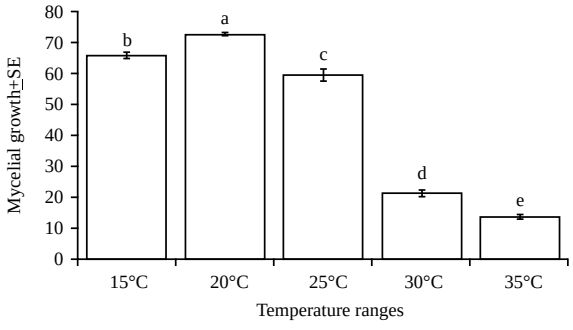

Fig. 6: Effect of temperature on mycelial growth (mm) of *N. chrysea* at 7 dpi

Value represents Mean±Standard error of nine replications, significant differences are presented as alphabet on the top of the bar and same letter do not differ significantly at 5% level of significance (DMRT)

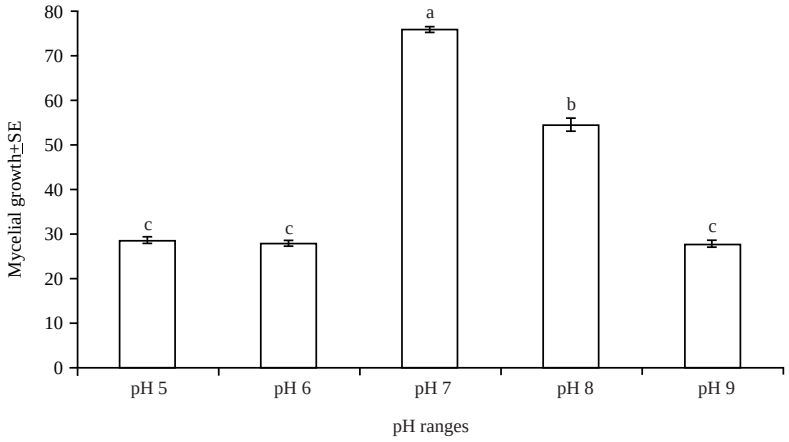

Fig. 7: Effect of pH on mycelial growth (mm) of *N. chrysea* at 7 dpi

Value represents Mean ± Standard error of nine replications, significant differences are presented as alphabet on the top of the bar and same letter do not differ significantly at 5% level of significance (DMRT)

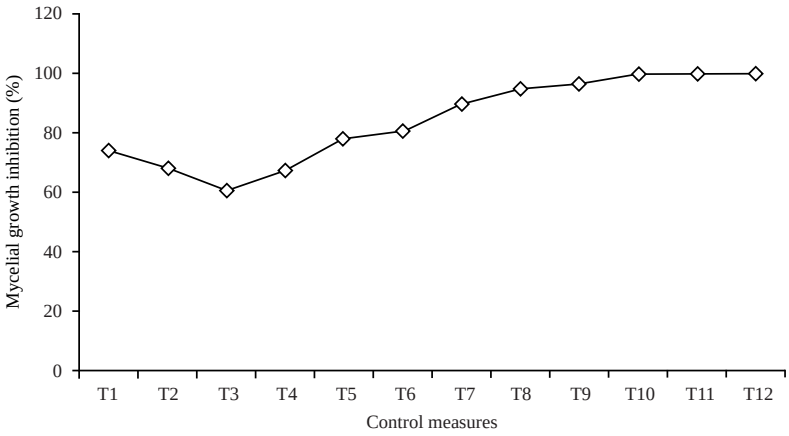

Fig. 8: Effect of antagonistic fungi and fungicides on the mycelial growth inhibition (%) of *N. chrysea* at 7 dpi

T1: *T. reesei*, T2: *T. harzianum*, T3: *T. asperellum*, T4: Ridomil (250 ppm), T5: Ridomil (500 ppm), T6: Ridomil (750 ppm), T7: Tilt (100 ppm), T8: Tilt (250 ppm), T9: Tilt (500 ppm), T10: Amistar (100 ppm), T11: Amistar (250 ppm) and T12: Amistar (500 ppm)

and development of tested fungus was determined through this part of the experiment. The experimental plates were incubated at five different pH viz., 5, 6, 7, 8 and 9 at room temperature. The greatest (75.5 mm) mycelial growth of *N. chrysea* was measured at pH 7 which was statistically significant with other treatments, the growth rate of this fungus with increasing pH level in Fig. 7. Results are in agreement with previous findings of El-Gali[17], who recorded an optimum growth of *Pestalotiopsis palmarum* and *Pestalotiopsis guepinii* at pH 7. However, McQuilken and Hopkins[24] reported that three selected isolates of *Pestalotiopsis* sp. grew well at pH 5.5.

Efficacy of *Trichoderma* spp. and synthetic fungicides against the fungal pathogen: The antagonistic effect of three bio-control agents *Trichoderma reesei*, *T. harzianum* and *T. asperellum* were evaluated on *N. chrysea* at 7 dpi in room temperature. The maximum (73.68%) inhibition of mycelial growth of the fungus was found due to *T. reesei*, followed by 68.23% in *T. harzianum* treatment, while the minimum (64.34%) inhibition was recorded by *T. asperellum* in Fig. 8 and Fig. 9a-c. The findings of the present experiment are supported by Bhadra *et al.*[25] who reported that *Trichoderma* spp. is known to inhibit pathogenic fungal invasion via mycoparasitism, antibiosis, competition and lysis of fungal hyphae. Besides, Kumhar *et al.*[26] tested *Trichoderma viride* and *T. asperellum* against the mycelial growth of *Pestalotiopsis theae* and found that these antagonistic fungi inhibited the growth of the fungus.

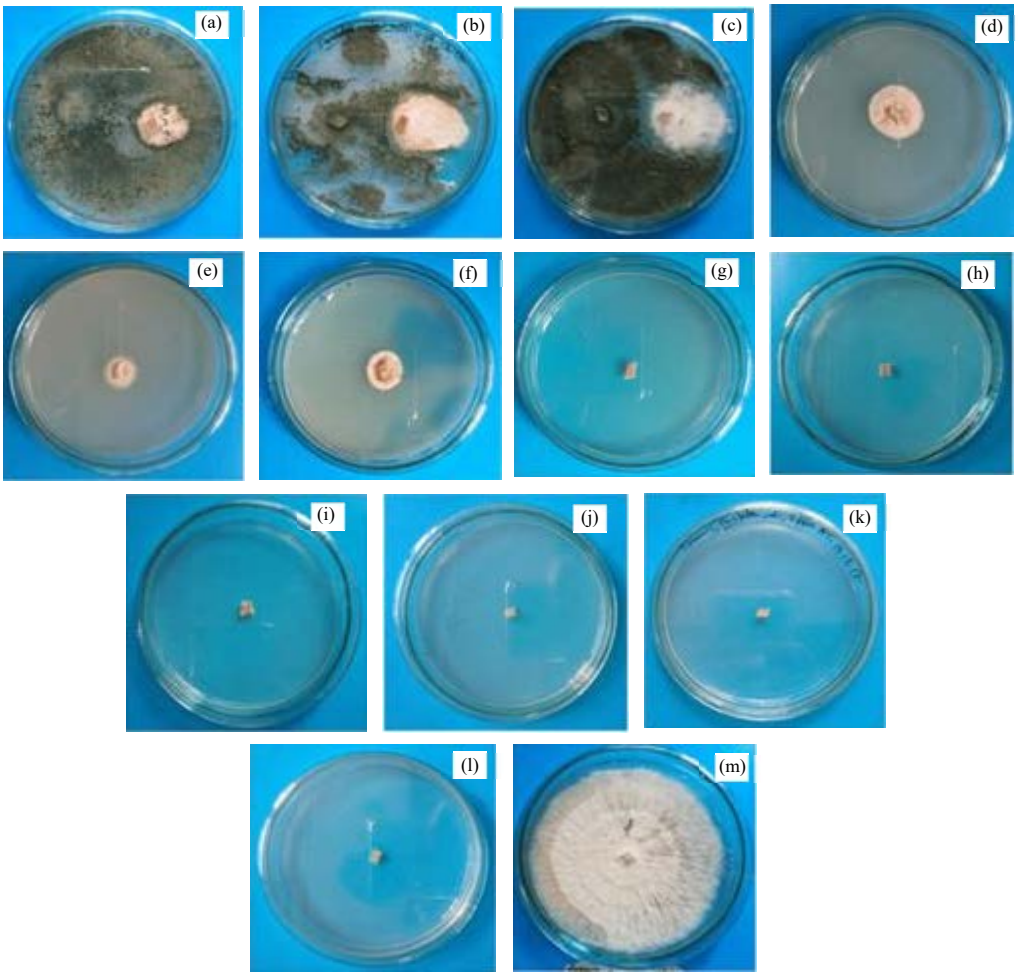

Fig. 9 (a-m): Effect antagonistic fungi and of fungicides on the mycelial growth inhibition of *N. chrysea* at 7 dpi, (a) *T. reesei*, (b) *T. harzianum*, (c) *T. asperellum*, (d) Ridomil (250 ppm), (e) Ridomil (500 ppm), (f) Ridomil (750 ppm), (g) Tilt (100 ppm), (h) Tilt (250 ppm), (i) Tilt (500 ppm), (j) Amistar (100 ppm), (k) Amistar (250 ppm), (l) Amistar (500 ppm) and (m) Control

In our experiment, complete (100%) mycelial inhibition of *N. chrysea* was observed due to all of the concentrations (100, 250 and 500 ppm) of Amistar top (Azoxystrobin plus Difenoconazole) in Fig. 8 and Fig. 9j-l, followed by 96% and 94% inhibition by 500 and 250 ppm of Tilt (propiconazole) in Fig. 8 and Fig. 9g-i. These results are supported by Nithyameenakshi *et al.*[27] cited that fungicide Amistar (Azoxystrobin 25% EC) was effective against grapevine diseases caused by *Plasmopara viticola*, *Uncinula necator* and *Gloeosporium ampelophagum*. Shamsi *et al.*[28] reported that Tall 25 EC (active ingredient: Propiconazole) was found to inhibit the vegetative growth of *Pestalotiopsis guepinii*, causal agents of anthracnose disease of *Senna alata* L. Besides, Chowdhury *et al.*[29] cited that propiconazole completely restricted the mycelial growth of several pathogenic fungi including *Pestalotiopsis guepinii*. Hossen *et al.*[30] also reported that chili seeds treated with Tilt 250EC (0.2%) were reduced the incidence of fungal association namely-*Aspergillus* spp., *Curvularia lunata*, *Colletotrichum capsici* and *Fusarium* spp. Billah *et al.*[31] cited that substantial mycelial inhibition of *Curvularia lunata* was achieved by propiconazole treatment in lab bioassay.

CONCLUSION

Leaf diseases are a major concern in strawberry cultivation. This article focuses on the identification, growth characteristics and *in vitro* management of *Neopestalotiopsis chrysea* causing leaf spot disease of strawberries. Present showed that the fungus had a specific preference to grow on

suitable fungal culture media, favourable temperature, pH and light conditions. Biological control agent *Trichoderma reesei*, chemical agents Amistar Top and Tilt 250 EC were found to be promising ways to restrict the fungus growth under laboratory bioassay. However, further pot and field trial is necessary to confirm it.

SIGNIFICANCE STATEMENT

This patulous study discovered the growth behaviour of *Neopestalotiopsis chrysea* depending on various factors, which will benefit the researchers to study fungal biology. *In vitro* disease management strategies will help the researchers to design and recommend an effective controlling disease to the growers. Thus, this comprehensive study will make a substantial contribution to improving agricultural production.

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