Pathological characterization of *Corynesporacassiicola*isolates from traditional and non-traditional rubber growing areas

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Abstract

Rubber (*Heveabrasiliensis*) is one of the major economically important estate crops andgeneratesthe third largest export income of Sri Lanka. Rubber plantations established mostly in Wet Zone and certain regions in Intermediate Zone and the cultivated areas are known as traditional areas.However, presently rubber cultivation has been expanded to the dry zone of country and the cultivated areas are known as non-traditional areas. *Corynesporacassiicola* is the most destructive foliar pathogen of the rubber plantcausing *Corynespora*leaf fall disease (CLFD) and the disease has caused a major devastation in rubber industry resultingin a remarkable economic loss. This study aimedto determine the variability of*C. cassiicola* isolates from traditional and non-traditional rubber growing areas using pathological factors. Ten isolates of *C. cassiicola*which had been isolated from diseased leaves of different clones grown in traditional (five isolates) and non-traditional (five isolates) areas were used for characterization.Variability inpathogenicity, temperature sensitivity,growth rate,conidia production,fungicide sensitivity to two fungicides; mancozeb and carbendazim and toxin production were examined. Data were statisticallyanalyzedand the final analytical output revealed a statistically significant difference ($p < 0.05$) between the isolates, but not between two geographical regions for all parameterstested except for toxin production and sensitivity to carbendazim. Though isolates of *C.cassiicola*showsignificant difference in pathological factors among isolates irrespective of geographical location, they do not behave differently in different climatic regions.

Keywords: Corynesporacassiicola, CLFD, toxins, traditional and non-traditional areas

1. Introduction

Rubber (*Heveabrasiliensis*) is the major source of natural rubber and cultivated commercially as a plantation crop. Rubber has a high demand in local and export market as sheet rubber, crepe rubber and rubber wood which make rubber as income generatingplantation crop in Sri Lanka. Rubber industry is the third largest export income of the country (EDB blog, 2015).

Earlier rubber plantations were mainly confined to the Wet Zone of the country; Kaluthara, Kegalla, Gampaha, Rathnapura, Colombo, Mathara and Galle, and Intermediate Zone; Kurunegala,Matale and Moneragala administrative districts and rubber growing areas are called as traditional areas. Expansion of rubber cultivation to the Dry Zone; Monaragala, Vauniya, Hambanthotaand Ampara administrative district was commenced in 2003(Iqbalet. al, 2010) andthese areas under rubber plantations are calledas non-traditional areas (Rodrigo, 2007). Continuous planting resulting in physicaland chemical property exhaustion of soil,change in land use pattern, urbanization and industrializationand high annual

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rainfall being a constraint for latex harvesting have led the expansion of rubber cultivation to new potential sites in non-traditional areas (Nugawela, 2002).

Both traditional and non-traditional rubber cultivations are affected by many biotic and abiotic factors. *Corynesporacassiicola*, a biotic factor causes delay in growth and maturity in immature trees and marked reduction in latex yield of mature trees (Jingiet al., 2007).*Corynespora*leaf fall disease (CLFD) causes a significant impact on rubber cultivations in Asian and African region(Jayasinghe, 2000) and the first CLFDepidemic in Sri Lanka was reported in late 1980's. The characteristic symptom of the fungus on rubber leaves is known as "railway track-like lesions". *C.cassiicola*isolates show different characteristics in morphology, pathogenicity, toxin production and spore production even though isolates are obtained from the similar agro climatic zone (Jayasinghe, 1999;Fernando etal., 2012).The diversity and variability of *C.cassiicola*are prime important factors to be considered in the development of resistant clone (Fernnadoet al., 2009).Occurrence of CLFD is reported in rubber plantations in non-traditional areas (unpublished data).Possibility of emerging different physiological races of*C.cassiicola*in different climatic zoneshas not been explored in Sri Lanka. This study aimed to determine the variability and pathological factors of *Corynesporacassiicola* isolates from traditional and non-traditional rubber growing areas.

2. Methodology

2.1*Collection of isolates*

Ten isolates of*C.cassiicola* which had been isolated from symptomatic leaves of different clones grown in traditional and non-traditional areas were obtained from Rubber Research Institute of Sri Lanka. Ten isolates comprisedfive from traditional areas and five from non-traditional areas (Table 1).

Table 1: Rubber clones, location and geographical regions of *Corynesporacassiicola* isolates.

Isolate No.	Rubber clone	Location	Geographical region
TA1	RRISL 110	Galewaththa	Traditional
TB2	RRISL 200	Batuwita	Traditional
TC3	RRISL 202	Kuruwita	Traditional
TD4	RRISL 217	Neboda	Traditional
TE5	RRIC 222	Batuwita	Traditional
NTF6	RRIC 121	Thelulla	Non – traditional
NTG7	RRIC 121	Padiyathalawa	Non – traditional
NTH8	RRIC 121	Padiyathalawa	Non – traditional
NTI9	Seedlings	Monaragala	Non – traditional
NTJ10	Seedlings	Monaragala	Non – traditional

2.2 Pathogenicity

Pathogenicity of the 10 isolates was tested using the detached leaf method (Brown and Soepena, 1994). Aqueous spore suspensions were prepared using tenday old cultures of the isolates grown on PDA. Conidial density was enumerated using a haemocytometer and inoculum size was adjusted to 1.0×10^4spores/mL.Copper brown leaves of a resistant clone (RRISL 121) and a susceptible clone (RRISL 201) were inoculated with respective spore suspensions. Six leaves (replicates) were usedfor each isolate. Each leaf was inoculated by placing six drops of spore suspension (10 µL)on both sidesof the mid rib of the upper surface of the leaves. Sterile distilled water (SDW) was used as the control. After inoculation, leaves were kept in a humid chamber under approximately 100% RH for 72 h.Lesion sizes were measured along the two diameters.

2.3 Temperature sensitivity

To determine the effect of temperature on growth of each isolate, 5.0 mm mycelial plugs wereplaced centrally on PDA medium in 9.0 cm Petri dishand incubated at 5, 10, 15, 20, 25, 30, 35 and 40^{o}C for 10 days, and the colony diameter of each isolate was measured along two lines perpendicular to each other throughout the incubation period. Five replicates were used for each isolate.

2.4Colony growth rate on PDA

Mycelial plugs (5mm diameter) were placed centrally on PDA medium in 9.0 cm Petri dish. Five replicates were used for each isolate. Cultures were incubated at room temperature (28±^{o}C)for 10 days. The colony diameter of each isolate was measured along two lines perpendicular to each other throughout the incubation period.

2.5Conidia production

Mycelial plugs (5mm diameter) were placed on PDA medium and cultures were incubated at room temperature for 10 days and conidia production (No. of conidia/cm^2) was enumerated for each isolate.

2.6 Fungicide sensitivity

Poison food technique was performed. Eight-day old mycelial plugs (5mm diameter)were placed on PDA medium containing different fungicide concentrations. Carbendazim, 2, 3 and 4 ppm andmancozeb, 50, 100, 200, 400, 800, 1000 and 1600 ppm concentrations were in the test medium. Three replicates were used for each concentration. Plates were incubated at room temperature for 10 days. The colony diameter of each isolate was measured along two lines perpendicular to each other throughout the incubation period. Following formula was used calculate percentage of the inhibition.

$$I = 100\,(C - T)\,/\,C \qquad (1)$$

Where:

I = Percentage of the inhibition
C = Growth in the control without fungicide
T = Growth in the treatment

2.7Toxin production

Toxic metabolite was extracted using Cazpek'sDox Broth (CDB). Two of the most spore producing isolates from each group (traditional and non-traditional) were selected for the toxin assay.

Leaf wilt bioassay

Apple green leaves from the susceptible (RRISL 201) and resistant (RRISL 121) clones were used to detect the toxin activity. Broth cultures were prepared using CDB (100 mL)inoculated with 10 day old three mycelial plugs (5 mm) and incubating for different time intervals. After the particular incubation period, crude toxin was filtered through sterile Whatman no.1 filter papers.

Fresh leaves were excised under water and immediately dipped in 5 mL of crude toxin filtrate and leftfor 24 h at room temperature. Three replicates were used for each isolate. The control leaves were dipped in 5 mL of sterile CDB. After 24 hours of incubation, leaves were examined for theintensity of wilting and categorized on a visual basis; Group I=no reaction, Group II=mild wilting, Group III= moderate wilting, Group IV=severe wilting. Leaf-wilt bioassay was used to determine the optimum incubation period for toxin extraction where severe wilting is resulted.

Leaf puncture bioassay

Toxinswere extracted as described in above and incubated at room temperature for 12 days. After the incubation period, the toxin was extracted filtering through sterile Whatman no.1 filter papers and then using milipore membranes.

Apple green leaves fromclones RRISL 201(susceptible) and RRISL 121(resistant) were collected for leaf puncture bioassay. Six leaves (replicates) were used foreach isolate. Each leaf was inoculated by placing six drops of the filtered toxic metabolite (20 µL) on both sides of the mid rib of the upper surface of the leaf. Sterile CDB was used as the control. After inoculation leaves were kept in a humid chamber under approximately 100% RH for 72 h. The lesion size was measured along two diameters.

2.8Statistical analysis

Data for pathogenicity, temperature sensitivity, growth rate, conidia production, fungicide sensitivity and toxin production were obtained using CRD (Complete Randomized Design). Data were analyzed using PC SAS PROC GLM version 9.1 (SAS institute, Cary, North Carolina) and the mean separation was done by DMRT (Duncan's Multiple Range Test) at $p<0.05$ level.

3. Results

3.1. Pathogenicity

Disease severity was higher in RRISL 201 infected by TA1 compared to other isolates irrespective of growing region exhibiting highest pathogenicity in the susceptible clone (Figure 1).

In the susceptible clone, RRISL 201,richmycelial masses were developed from the selected isolatesafter 72 h of incubation. Isolate NTI9 showed lowest pathogenicity in the resistant clone RRISL 121 while a remarkably higher pathogenicity was observed in clone RRISL 201.Different fungal isolates showed a significant variation ($p<0.05$) on pathogenicity. However, based on Duncan's multiple grouping analysis, pathogenicity of traditional isolates and non-traditional isolates was not significantly different.

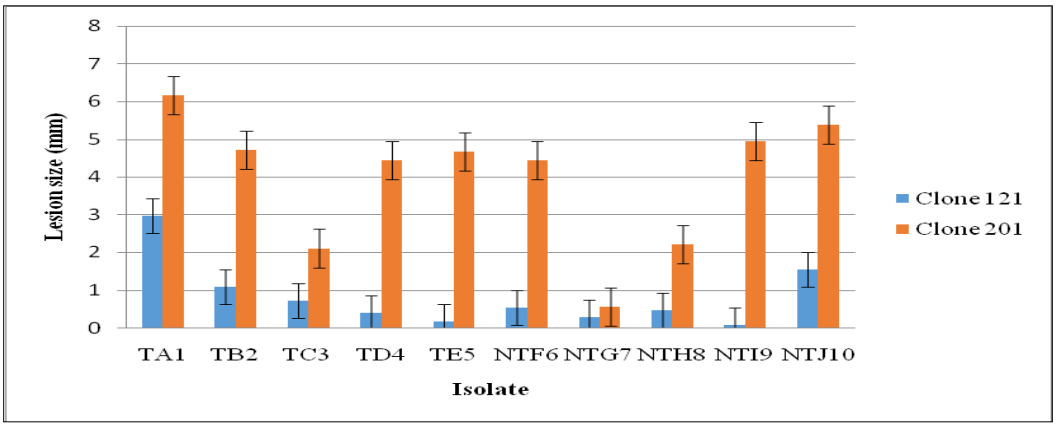

Figure 1: Mean lesion size vs. *C. cassiicola* isolates for pathogenicity. Values are means of six replicates. Error bars represent standard error of means.TA1 – TE5: Isolates from traditional rubber growing areas. NTF6 – NTJ10: Isolates from non-traditional rubber growing areas.

3.2Temperature sensitivity

Growth of all the isolates was inhibited at 5°C and 40°C (Figure 2: A and B).

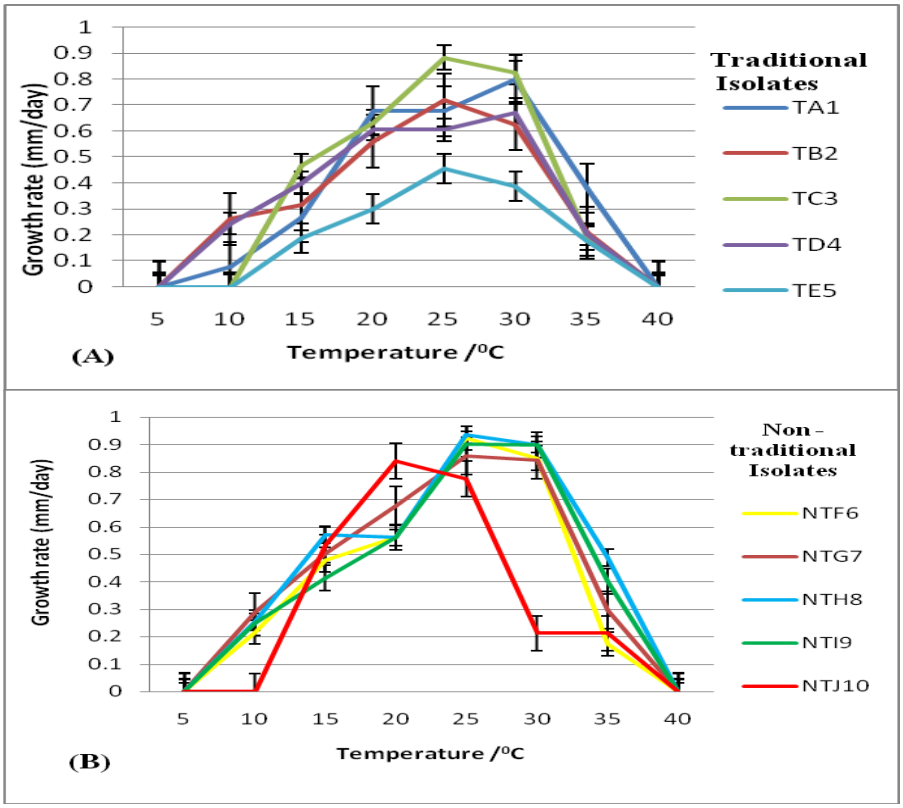

Figure 2: Growth rate of traditional and non- traditional isolates *C. cassiicola* in different temperatures. Values are means of three replicates. Error bars represent standard error of means. TA1 – TE5: Isolates from traditional rubber growing areas. NTF6 – NTJ10: Isolates from non – traditional rubber growing areas.

The optimum temperature for the growth of isolates TA1 and TD4 was 30ºC. And for every other isolate, the optimum temperature was recorded as 25ºC.Different fungal isolates showed a significant variation ($p<0.05$) on temperature sensitivity.However, based on Duncan's multiple grouping analysis, temperature sensitivity of isolates from traditional isolates and non-traditional isolates was not significantly different.

3.3Growth rate

Isolate TA1 showed the maximum growth rate while TE5 showed the minimum growth rate (Figure 3). Isolates from non-traditional areas showed more or less uniform growth and the growth rates are non – significant. Different fungal isolates showed a significant variation ($p<0.05$) on the growth rate.However, based on Duncan's multiple grouping analysis, the growth rate of different isolates from traditional areas and non- traditional areas was not significantly different.

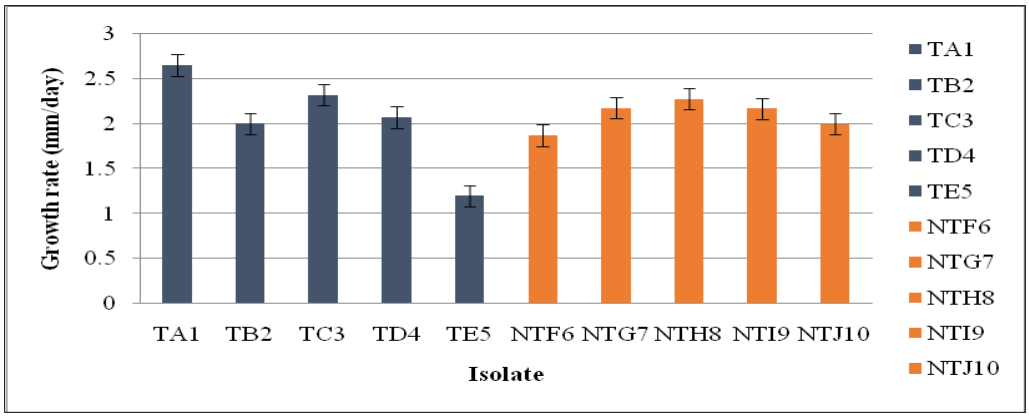

Figure 3**:** Growth rate of *C. cassiicola* isolates from traditional and non- traditional areas. Values are means of five replicates. Error bars represent standard error of means. TA1 – TE5: Isolates from traditional rubber growing areas. NTF6 – NTJ10: Isolates from non-traditional rubber growing areas.

3.4 Conidia production

Isolates NTF6 and NTH8 showed the highest conidia production while isolate TD4 showed the lowest conidia production (Figure 4).

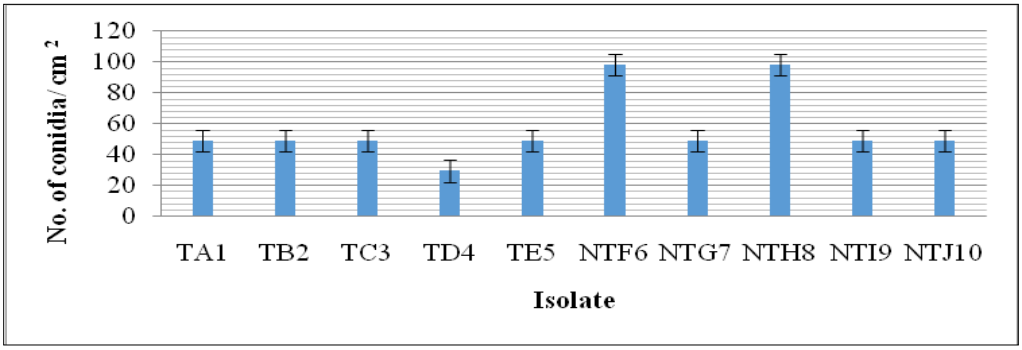

Figure 4**:** Conidia production per area of traditional and non- traditional *C. cassiicola* isolates**.** Values are means of three replicates. Error bars represent standard error of means. TA1 – TE5:Isolates from traditional rubber growing areas. NTF6 – NTJ10: Isolates from non-traditional rubber growing areas

Rest of isolates produced more or less same amount of conidia. Different fungal isolates showed a significant variation ($p<0.05$) on conidia production. However, based on Duncan's multiple grouping analysis, conidia production in traditional isolates and non-traditional isolates was not significantly different.

3.5 Fungicide sensitivity

Variability in the sensitivity to the fungicide, mancozeb

Isolate NTI9 exhibited the minimum percentage inhibition for all concentrations. The isolates; TA1, TD4 and NTF6 expressed the highest percentage inhibition for 50ppm, 100–800ppm and 1000–1600ppm respectively (Figure 5).Different fungal isolates showed a significant variation ($p<0.05$) on sensitivity to the fungicide, mancozeb. However, based on Duncan's multiple grouping analysis, sensitivity of isolates to mancozeb from traditional isolates and non – traditional was not significantly different.

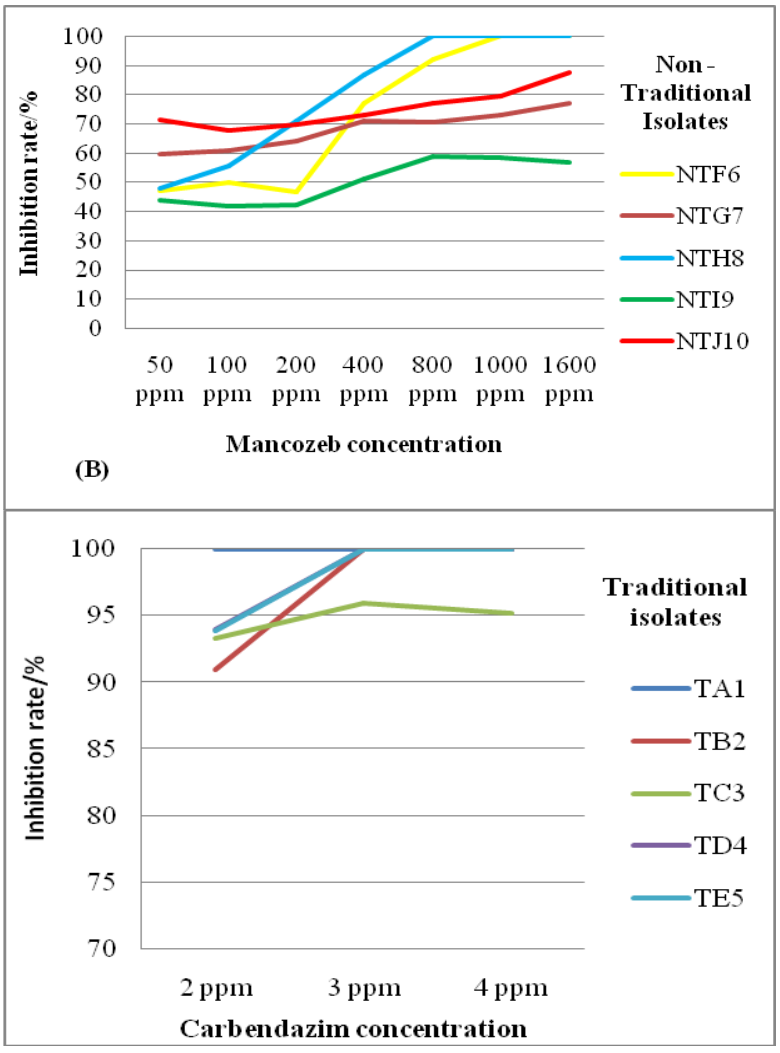

Figure 5: Percentage inhibition of traditional and non- traditional *C. cassiicola* isolates in different concentrations of mancozeb. Values are means of three replicates. Error bars represent standard error of means. TA1 – TE5: Isolates from traditional rubber growing areas. NTF6 – NTJ10: Isolates from non-traditional rubber growing areas

Variability in the sensitivity to the fungicide, carbendazim

Growth of ten isolates wastotally inhibited at 4 ppm carbendazim concentration while 95% of growth inhibition observed in TC3 (Figure 6).Different fungal isolates didnot show a significant variation ($p<0.05$) on sensitivity to the fungicide,carbendazim.

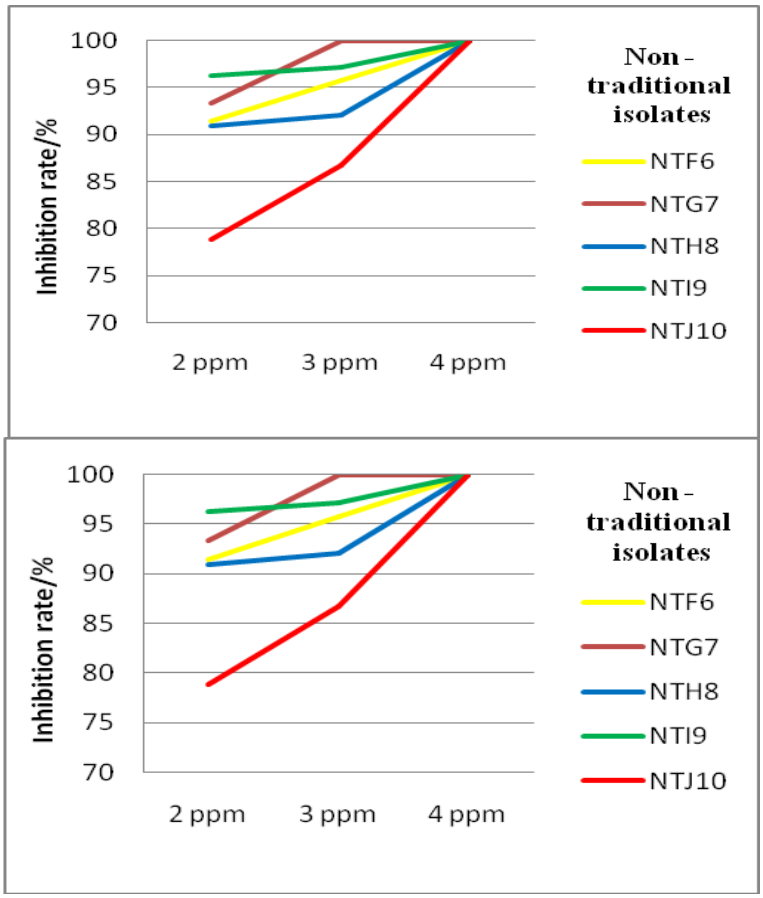

Figure 6**:** Percentage inhibition of traditional and non-traditional *C. cassiicola* isolates in different concentrations of carbendazim. Values are means of three replicates. Error bars represent standard error of means. TA1 – TE5: Isolates from traditional rubber growing areas. NTF6 – NTJ10: Isolates from non-traditional rubber growing.

3.6. Toxin production

Results of the leaf wilt bioassay showed sufficient toxin production in CDB broth inoculated with mycelial plugs after 12 days of inoculation. Therefore, leaf puncture bioassay was done using 12 day old broth culture. Toxin activity indices in the susceptible clone were higher compared to the resistant clone (Figure 7). Different fungal isolates showed a significant variation ($p<0.05$) on toxin activity.

The toxin activity of the selected isolates demonstrated two different statistics. In the resistant clone (RRISL 121) toxin activity of isolate TA1 and isolate NTF6 expressed a statistically significant difference. Duncan grouping confirmed this difference. Isolate NTF6 expressed more toxin activity than the isolateTA1.

However, in the susceptible clone (RRISL 201), such significant difference could not be observed in the toxin activity by the two isolates. Duncan grouping confirmed the absence of difference. However, according to Duncan grouping, the toxin activity of the isolate TA1 in RRISL 201was slightly higher than isolate NTF6.

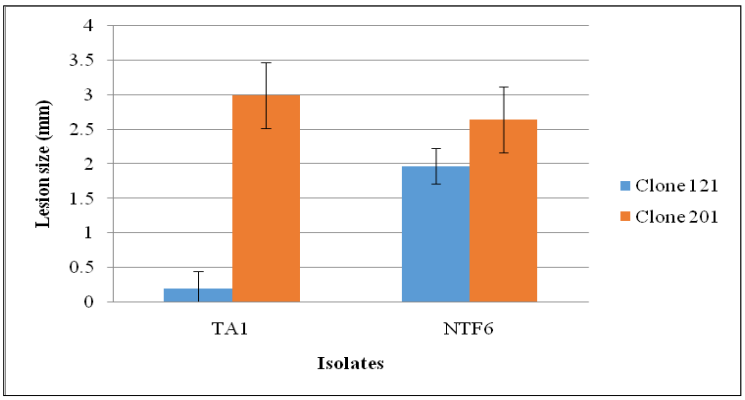

Figure 7**:** Mean lesion size vs. *C. cassiicola* isolates for toxin activity. Values are means of three replicates. Bars represent standard error of means. TA1 – TE5: Isolates from traditional rubber growing areas. NTF6 – NTJ10: Isolates from non – traditional rubber growing areas.

4. Discussion

Presently, rubber is grown as plantation crop in traditional and non-traditional areas of the country. Destructive foliar disease *Corynespora* leaf fall occurs in non-traditional areas too. However, characterization of *C. cassiicola* isolates from new locations in the Dry Zone has not been done which is important in designing management strategies. Present study focused on investigating variability of *C.cassicola* isolates from two different geographical regions.

Results reveal that the pathogenicity of *C.cassicola* isolates varies between susceptible and resistant clones. The same results were found in a previous study which reports variability of pathogenicity among isolates of *C.cassicola* (Cheeet al., 1987). Pathogenic effect was higher in all isolates in the disease susceptible clone RRISL 201 compared to resistant clone RRISL 121. Although there is a significant difference between the isolates, difference in pathogenicity between the two geographical regions is non-significant.It is evident that a significant variation in the growth pattern of the selected isolates of *C.cassiicola* and the findings agree with the observations of Fernando et.al (2009) where all *C.cassiicola*isolates examined, had shown similar growth patterns on PDA witha significant variation in the growth rates.Present study based on two different climatic zones reveals that there is no significant difference in growth rate between the two geographical regions.Results revealed that the optimum temperature for the growth of *C.cassiicola*isolateslie in the range of 25 -30ºC. Previous studies also reported the optimum temperature is between 25-30ºC for *C. cassiicola*mycelial growth, and 30ºC is the most favorable temperature (Chunxiaet al., 2010). However, isolates didnot express a significant difference in the temperature sensitivity between the two geographical regions where temperature difference exists over the year with different monsoons.

Variation in conidia production under controlled environmental conditions was observed in test isolates irrespective of origin of isolates. Similar finding is reported; typical slender, needle shaped conidia and significant variation in conidia production in different *C.cassiicola* isolates (Schlubet

al.,2012). Results of the present study reveal a slight variation in production in different isolates indicating different climatic conditions do not affect conidia production. Hence, present findings provide the information on the similar potential of secondary infection in both geographical regions.

Mancozebis the mainly used fungicide to control CLFD. But due its adverse effects on the environment, Rubber Research Institute is now promoting carbendazim, which has shown low toxicity levels. Therefore, both of fungicides were evaluated inthis study. It was found that both mancozeb and carbendazimare effective fungicides against tested isolates from both regions and effective concentrations of different fungicides are totally different. The results of the present study are agreeable with the results of previous study on mancozeb and carbendazimagainst *C.cassiicola*from rubber plants reported by Fernando et al., (2010).

When comparing both fungicides, carbendazim showed drastically low inhibition concentrations. Since inhibitory effect of carbendazim on growth of all tested isolates is higher compared to mancozeb, carbendazim can be recommended as effective fungicide against *C. cassiicola*isolates in the country. In the chemical control of *Corynespora* leaf fall disease, carbendazim is a promising chemical agent to achieve successful growth suppression with minute amounts. Further, it provides direct economic advantage as an effective product. On the other hand, environment pollution due to excessive amounts of chemicals can be minimized with application of fungicides in reduced amounts. However, it is important to evaluate its possible negative effectsbefore applying it in large scale plantations.Susceptibility or resistance of a clone is determined by the ability of a pathogen to establish in that particular clone. In nature, toxic metabolite production is commenced after the establishmentof the pathogen inside the plant tissue. In this study toxins were applied externally for the bioassay, and lesion development was taken as the effect of toxin on pathogenic activity of *C.cassiicola* on susceptible and resistant clones.

When considering the toxin activity between the two geographical regions, a significant difference was observed between the two geographical regionsin both susceptible and resistant clones. In a previous study, it was observed that there is variability in the virulence of different *C.cassiicola* isolates. This difference was resulted by a gene called *Cas*, which is responsible for the production of the toxin, cassiicolin (Reshmaet al., 2016). Pathogenicity of *C.cassiicola* in *Corynespora* leaf fall disease of rubber is related to a host selective toxincassiicolin (Breton etal., 2000). It is evident that pathogenicity factor, cassiicolin production rate of individual isolates determines the degree of pathogenicity of individual isolate in different geographic regions. Prevailing climatic conditions in non-traditional areas favors considerable amount of toxin production even in resistant clone RRISL 121. When in view of these results, it is apparent that Hevea clone might play a role in the different manner in toxin activity of isolates from different geographical regions.

Present study shows there is no remarkable difference between the isolates from two geographical regions. This may be due to the climatic difference between two regions not being diverse enough for the emergence of new physiological races. Moreover, since similar *Hevea* clones are cultivated in both regions probability of developing altered pathogenic factors within a short duration is low.

5. Conclusions

Present study shows there is no remarkable difference between the isolates from two geographical regions. This study reveals that carbendazim is a promising fungicide against *Corynesporacassiicola* isolates found in different regions and the fungicide, carbendazim can be used in CLFD management in traditional and non-traditional rubber growing areas of the country.

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