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***Research Paper***

# Genetic Diversity Detected by Vegetative Compatibility Test among Different isolates of *Cylindrocladium quinqueseptatum* Causing Leaf and Twig Blight in *Eucalyptus sp.*

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***Abstract -*** *The Cylindrocladium quinqueseptatum is soil borne pathogen causing leaf and twig blight in Eucalyptus nurseries, has a wide host range, common in Europe and Asia. Under present investigation fifteen isolates were recorded from diseased tissues of Eucalyptus from North Indian states viz, Punjab, Haryana, Himanchal Pradesh, Uttarakhand and Uttar Pradesh. These isolates were categorised in five different VCGs (Vegetative Compatibility Groups) on the basis of vegetative compatibility test. Out of 15 isolates studied, 8 isolates showed free intermingling with maximum combinations and placed under VCG1 and indicates the vegetative compatibility. Four isolates grouped into VCG2 which showed tuft formation, a kind of non-self fusion and indicates incompatibility. Three isolates 294, 145 and 138 respectively placed under VCG3, VCG4 and VCG5 on the basis of gap reaction, barrage reaction and line gap reaction and showed incompatibility. Based on above findings, it was concluded that vegetative compatibility test can be used for studying diversity and taxonomy of the fungi.*

**Keywords:** *Cylindrocladium quinqueseptatum,* VCGs (Vegetative Compatibility Groups), Anastomosis, Vegetative incompatibility.

## Introduction

The genus *Eucalyptus* is widely cultivated for socio-economic aspects in India with an average annual productivity of *Eucalyptus* 20 times that of natural forests putting it at a higher rank in terms of biomass production [1]. According to FAO report, 1979- in 1955, the total area under *Eucalyptus* plantations that was estimated about 700,000ha worldwide rose to 4000,000 hectares within 25 years. Leaf and twig blight of *Eucalyptus* caused by *Cylindrocladium quinqueseptatum* is a serious disease affecting *Eucalyptus* in nursery and plantations in India. In North India, the disease has been found to be pre-dominant in the different parts of Punjab, Haryana, Himanchal Pradesh, Uttarakhand and Uttar Pradesh. *Cylindrocladium sp*. is also widespread in Europe and South-East Asia [1]. In high rainfall areas *C. quinqueseptatum* causes severe mortality in seedlings of *Eucalyptus* by affecting its root. Sub-lethal infections can result in stunting and chlorosis or top dieback, with poor growth rate. Earlier reports distinguished 39 *Cylindrocladium sp.* out of these, 24 were listed as pathogen of *Eucalyptus* and 15 of these have been found in South East Asia [2]. So for the India's concern to control the disease over the fungus is very challenging. The idea reflects as study on this fungus in order to understanding variability in pathogen's population, *C. quinqueseptatum* isolates belonging to various geographic locations of India were characterized for their vegetative compatibility.

*C. quinqueseptatum* causing diseases in forest nurseries and eucalyptus plantations was found to be associated with more than 25 indigenous host species, which reveals the adaptability of the pathogen in different ecosystems. The isolates of *Cylindrocladium quinqueseptatum* from cashew, clove and eucalyptus are cross inoculable [3] but no report persist on the study of intraspecific variability in pathogenic fungus, *Cylindrocladium quinqueseptatum.* Little can be achieved by modifying cultural practices, as the disease is favoured by high humidity and the pathogen perinates in the soil. Chemotherapy is a costly affair and unfragmatic in plantations. Biological control is often erratic and its efficacy is environment dependent. The only cost effective and efficient remedy is identifying and developing resistanthost germplasm against this fungus. As the pathogens have different population lines, it is of prime importance to identify and characterize them, so that representative of each population line can be used for screening and identifying resistant host germplasm. Vegetative compatibility test is an efficient technique

employed for this purpose. Vegetative compatibility tests have been utilized to investigate the occurrence of different isolates within defined geographical areas and to study the population structure of different pathogenic, saprophytic and ectomycorrhizal fungi [4].

## Material and Methods

Isolates of *C. quinqueseptatum* were kindly provided by Molecular Pathology Laboratory, Forest Research Institute (Dehradun) and maintained on potato dextrose agar (PDA) Himedia powder. After getting the pure cultures of all fifteen isolates, these were paired in 115 combinations in petri plates on PDA, by placing two mycelial discs (3mm) in biculture manner described under Biculture Test [5, 6]. At least triplicates were prepared for each pairing, and after 20 days incubation at $25^0$C the pairings were analyzed for macroscopic and microscopic mycelial reactions. These reactions were assigned to five categories VCG1 (Vegetative Compatibility Group 1), VCG2 (Vegetative Compatibility Group 2), VCG3 (Vegetative Compatibility Group 3), VCG4 (Vegetative Compatibility Group 4) and VCG5 (Vegetative Compatibility Group 5) as evidenced on the basis of free intermingling, tuft formation, gap reaction, barrage reaction and line gap reaction [6, 7].

Paired colonies merged uniformly or there was a slight mycelial thickening along the interaction zone, no dark or brown line appeared on the back of the PDA plate is considered as free intermingling which is of concern of compatible isolates (Figure 1A, 1B, 1C). Remaining four categories falls under the incompatible reactions. The occurrence of non-self-anastomosis was characterized by the aerial hyphae at the line of contact and considered as tuft formation (Figure 2A). The gap reaction was characterized by a wide gap between two paired colonies and two dark lines on the back of the PDA plate (Figure 2B). The line gap reaction was characterized by a narrow gap along the interaction zone, which looked like a dark line and there were one or two brown lines on the back of the PDA plate (Figure 2C). The barrage reaction did not form gap or line gap at the interaction zone and was characterized by a thick, white mycelial barrage, about 3-5mm wide between two paired colonies and a dark zone on the back of the PDA plate (Figure 2D).

Microscopic imaging of fusion events in *C. quinqueseptatum* was monitored by Micro Image Projection System (MIPS), the development of self anastomosis and non-self anastomosis in different mycelial isolates were seen under microscopy. Images were captured and edited using micro image projection system, which also allowed the conversion into JPEG format.

## Results

Fifteen isolates paired in 115 combinations on PDA showed different interaction behaviours as free intermingling, tuft formation, gap reaction, barrage reaction and line gap reaction (Table 1) which were classified as VCG1 (Vegetative Compatibility Group 1), VCG2 (Vegetative Compatibility Group 2), VCG3 Vegetative Compatibility Group 3), VCG4 (Vegetative Compatibility Group 4), and VCG5 (Vegetative Compatibility Group 5) (Table2). The development of hyphal interaction under microscopic assessment was detectable through compatible anastomosis bridge (Figure 1B, 1C). The compatible reactions were characterized by free intermingling which was found in about 41.73% (48 fusions, 115 combinations) of different combinations. There are 8 isolates (305, 119, 229, 165, 233, 222, 221 and 190) which showed maximum free intermingling reaction with other isolates and placed under VCG1. Incompatible reactions were considered on the basis of tuft formation (28.69%), gap reaction (13%), barrage reaction (9.56%), and line gap reaction (6.95%) between pairing of different isolates (Figure 2A, 2B, 2C, 2D). These reactions were detectable through hyphal by-passing (Figure 2E) and septum formation (Figure 2F) between contacted hyphae. Tuft formation was carried out by 33 combinations out of 115 (about 28.69% of total combinations), less number of combinations (13%) of different isolates showed gap reaction, followed by barrage reaction in 9.5% combinations of isolates. In 8 combinations (6.95%) line gap reaction was noticed (fig. 2D). Four isolates 232, 228, 235 and 269 categorised under VCG2 (Vegetative Compatibility Group 1) and indicates incompatibility between these isolates. Three isolates 294, 145 and 138 respectively placed into VCG3 (Vegetative Compatibility Group 1), VCG4 (Vegetative Compatibility Group 1) and VCG5 (Vegetative Compatibility Group 1) on the basis of incompatible reactions, gap reaction, barrage reaction and line gap reaction. In nutshell, on the basis of hyphal interaction between 115 different combinations of 15 isolates, approximately 41.7% pairing showed free intermingling interpreted as compatibility, remaining 58.3% combinations shows non-self fusion and detected as incompatible pairing.However pure cultures of these strains were used as control for each combination.

## Discussion

This study represents our understanding on the occurrence of vegetative compatibility between different isolates of *Cylindrocladium quinqueseptatum*. Biculture method for the detection of vegetative compatibility resulted that the 41.73% combinations of isolates were compatible which is indicated by free intermingling through the formation of anastomosis bridge (Figure 1B, 1C). Previously many other workers pointed out anastomosis formation in AM (Arbuscular Micorhizal) fungi [8,9,10]. Anastomosis (hyphal fusion) is a widespread phenomenon, and it may be considered one of the main morphogenetic event of fungal life cycle. Anastomosis by the means of mycelial network for communication, and nutrient translocation within fungal individual [11].

The detection of nuclei in hyphal bridges after self-anastomosis in all *Cylindrocladium quinqueseptatum* isolates confirms the occurrence of nuclear migration through the fusion pore developed during hyphal anastomosis[12,13,14,15]. Nuclear exchange following anastomosis may represent a fundamental mechanism allowing the maintenance of genetic diversity in the absence of sexual recombination [14,16,17]. Microscopic images of interacting hyphae between isolates allowed us to monitor the events leading to anastomosis formation, cell wall lysis

and protoplasmic mingling. Some non-self-fusion reactions *i.e.* tuft formation (28.69%), gap reaction (13%), barrage reaction (9.56%) and line gap reaction (6.95%), also observed during the hyphal interaction between isolates, which indicates incompatibility between isolates (Table1 and Figure 2A, 2B, 2C and 2D). Many workers support the occurrence of non-self fusions between hyphal interactions through microscopic studies [5, 6, 7].

The discrimination of co-specific isolates on the basis of their macroscopic and microscopic vegetative reactions *i.e*. compatibility (hyphal fusions between colonies or free intermingling) or incompatibility (lack of fusions, hyphal rejection, septum formation at contact zone and/or hyphae bypassing) *C. quinqueseptatum* isolates showed compatible responses only in intra-isolate pairing, whereas all inter-isolate pairing showed vegetative incompatibility, irrespective of their self anstomosing ability. In present study high percentage (41.73%) of anastomosis were occurred by showing free intermingling (48 fusions, 115 contacts) and grouped under VCG1 (Vegetative Compatibility Group 1). No anastomoses were occurred in pairings between germ lings of different isolates which showed tuft formation in 28.69% (33 fusions, 115 hyphal contacts), gap reaction in 13% (15 fusions, 115 hyphal contacts), barrage reaction in 9.56% (11 fusions, 115 hyphal contacts) and line gap reaction in 6.14% (8 fusions, 115 hyphal contacts) pairings so grouped into VCG-2 (Vegetative Compatibility Group 2), VCG-3 (Vegetative Compatibility Group 3), VCG-4 (Vegetative Compatibility Group 4) and VCG-5 (Vegetative Compatibility Group 5) respectively. The finding concludes that self and non-self-hyphal compatibility may play an important role in fungal life cycle, allowing either hyphal interconnection or protoplasm mingling within the same genet or the maintenance of individual genetic identity [5]. Fifteen isolates of *C. quinqueseptatum* belonging to different locations of India represent 5 different population lines. In future, breeding work while testing the varieties for disease resistance representative from each VCG (Vegetative Compatibility Group) should be tested to ensure proper screening of durable resistance in the germplasm.

## Conclusion

From our findings we can concluded that vegetative compatibility test is still reliable conventional method over molecular era and quite successful in detecting phylogeny of complex pathogenic fungi.

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**Table1: Description of compatibility and incompatibility among *Cylindrocladium quinqueseptatum* isolates**

| | 305 | 294 | 228 | 232 | 269 | 165 | 119 | 229 | 222 | 221 | 235 | 145 | 190 | 233 | 138 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 305 | * | TF | | | | FI | FI | FI | TF | | GR | LG | LG | FI | GR |
| 294 | | * | | | | TF | BR | | TF | LG | FI | TF | GR | TF | GR |
| 228 | TF | BR | * | BR | FI | | FI | FI | TF | | TF | TF | | TF | |
| 232 | | | FI | * | | | | TF | | GR | FI | | FI | FI | TF |
| 269 | FI | TF | | FI | * | GR | BR | | | BR | GR | GR | TF | BR | |
| 165 | | TF | | | TF | * | | FI | FI | BR | | FI | TF | | LG |
| 119 | GR | TF | | | LG | FI | * | FI | LG | TF | LG | | | FI | FI |
| 229 | | | | | | | | * | | FI | | TF | | FI | TF |
| 222 | | | | | | FI | | FI | * | TF | | GR | GR | FI | TF |
| 221 | | | | | | | | FI | | * | | | TF | | TF |
| 235 | | | | | BR | FI | | TF | | BR | * | TF | | FI | |
| 145 | | | | | | | | | | GR | | * | | GR | GR |
| 190 | TF | LG | FI | GR | BR | | FI | FI | TF | BR | FI | TF | * | TF | TF |
| 233 | | | | | | | | | | | | | | * | |
| 138 | | | | | | | | | | | | | | | * |

*-Control, FI-Free intermingling, TF-Tuft formation, GR-Gap reaction, BR-Barrage reaction, LG-Line gap reaction.

**Table 2**: **Five different VCGs containing compatible and incompatable isolates of *Cylindrocladium quinqueseptatum***

| **VCGs** | ***C. quinqueseptatum* isolates** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **VCG-1** | 305 | 119 | 229 | 165 | 233 | 222 | 221 | 190 |
| **VCG-2** | 232 | 228 | 235 | 269 | | | | |
| **VCG-3** | 294 | | | | | | | |
| **VCG-4** | 145 | | | | | | | |
| **VCG-5** | 138 | | | | | | | |

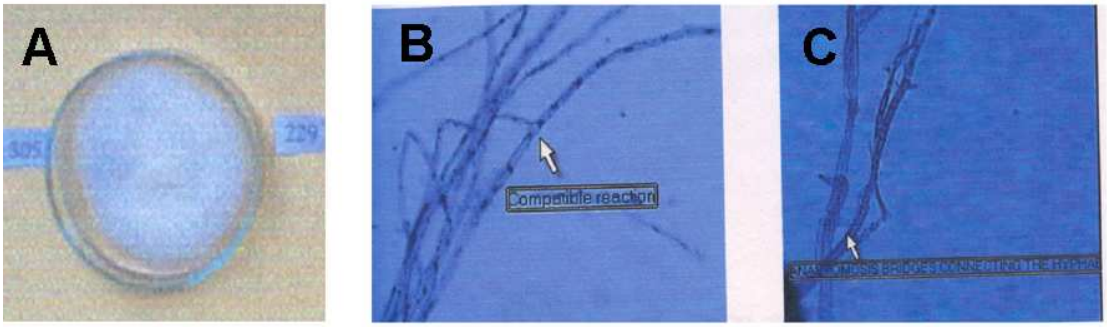


**Figure 1: Compatible isolates showing anastomosis bridge formation**
**A. Free intermingling, B. Anastomosis bridge, and, C. Anastomosis bridge**

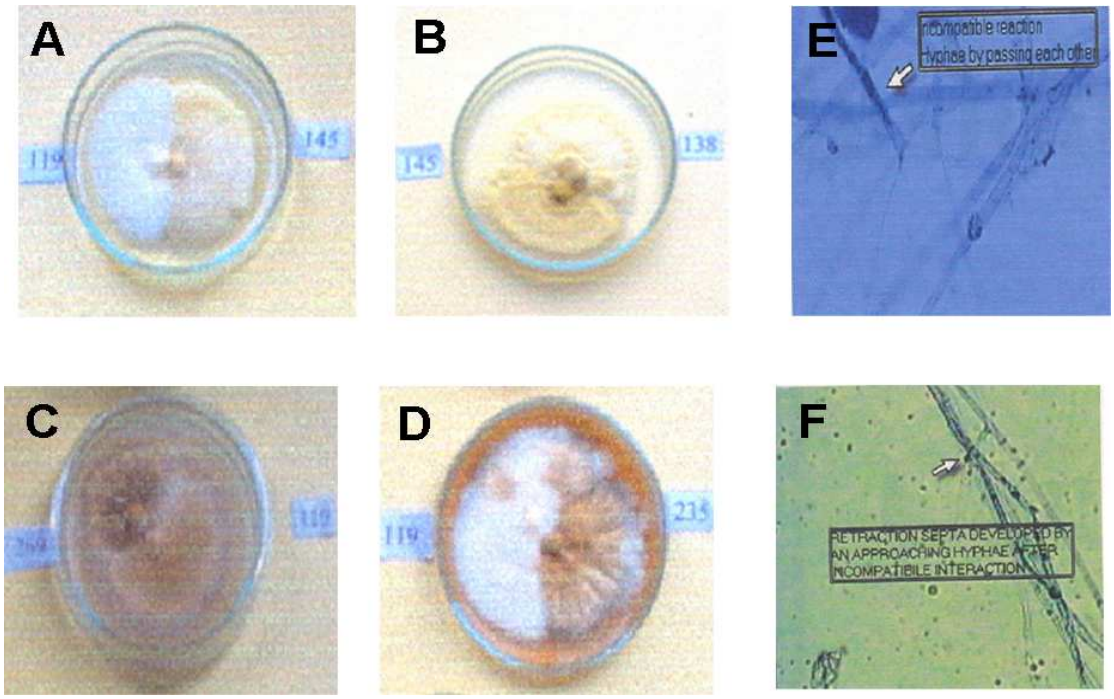


**Figure 2: Incompatible reactions showing Hyphal by-passing and retraction septa. A. Tuft Formation, B. Gap Reaction, C. Barrage Reaction, D. Line gap Reaction, E. Hyphal by-passing, and, F. Retraction septa**