Quorum sensing-mediated inter-specific conidial anastomosis tube fusion between Colletotrichum gloeosporioides and Colletotrichum siamense.

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Research

Keywords: Conidial anastomosis tube, Horizontal gene transfer, Quorum sensing, Genetic diversity, Hybrid vigour, Colletotrichum

DOI: https://doi.org/10.21203/rs.3.rs-54692/v1

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Abstract

Many plant pathogenic filamentous fungi undergo fusion of conidia through conidial anastomosis tubes (CATs), which is believed to facilitate horizontal gene transfer between species. We discovered a remarkable inter-specific conidial anastomosis tube (CAT) fusion between two important plant fungal pathogens Colletotrichum gloeosporioides and Colletotrichum siamense. In an *In vitro* assay, under no selection pressure, the inter-specific CAT fusion was preferred with higher frequency (25%) than intra-specific CAT fusion (11%). Different stages of CAT fusion *viz.* CAT induction, homing, and fusion were observed during this inter-specific CAT fusion. The CAT fusion was found to be higher in absence of nutrients and in the presence of physiological stresses. This CAT fusion involved a quorum sensing phenomenon, wherein the CAT induction was dependent on conidial density and the secreted putative quorum sensing molecules were found to be lipid based in nature. Movement of nuclei, mitochondria, lysosomes and lipid droplets were observed during the CAT fusion. Post CAT fusion, the resulting conidia gave rise to putative recombinants with variable colony characteristics as compared to their parental strains. Few recombinants showed variable AFLP banding pattern compared to their parental strains, thereby suggesting a possible genetic exchange between the two species through CAT fusion. The recombinants exhibited variable fitness under different stress conditions. Our study signifies possible role of inter-specific CAT fusion in generation of genetic and phenotypic diversity in these fungal pathogens.

Introduction

The *Colletotrichum* is a widespread genus voted as the eighth most important group of plant pathogenic fungi in the world (Dean et al., 2012). Among different species, *C. gloeosporioides* and *C. siamense* cause anthracnose in a wide range of host plants such as fruit, vegetable and ornamental plants (Cai et al., 2009; Freeman et al., 1996; Hyde et al., 2009; Jayawardena, 2018; Sharma et al., 2013). We have shown that there is a high level of genetic diversity in *C. gloeosporioides* isolates in India (Mehta et al., 2017). Our observation also corroborates with a series of studies published on genetic and pathogenic diversity of *C. gloeosporioides* (Denoyes-Rothan et al., 2003; Nova et al., 2011; Weeds et al., 2003). The possible mechanism of generation of such high genetic diversity may lie in meiotic recombination during sexual reproduction, however, it has been suggested that the sexual reproduction in *C. gloeosporioides* in nature would be very rare, if not absent (Weir et al., 2012). Similarly, no sexual stage for *C. siamense* has been demonstrated till date (Prihastuti et al., 2009).

Horizontal gene transfer (HGT) or horizontal chromosome transfer (HCT) may contribute to the high level of genetic variation found in fungi that lack sexual reproduction (Ishikawa et al., 2012; Mehrabi et al., 2011). The HCT and HGT have been considered to play an important role in fungal plant pathogens evolution (Rosewich and Kistler 2000; Walton 2000; Oliver and Solomon 2008; Rep and Kistler 2010; Mehrabi et al. 2011). The horizontal chromosome transfer (HCT) has been demonstrated in *C. gloeosporioides, Alternaria alternata* and *Fusarium oxysporum* (Akagi et al., 2009; He et al., 1998; Ma et al., 2010). Horizontal chromosome transfer (HCT) has also been demonstrated between two vegetatively incompatible biotypes of *C. gloeosporioides* co-cultured under laboratory conditions and this is also
thought to occur in nature (He et al., 1998; Manners and He, 2011); however, it is not very clear how exactly the HGT/HCT occurs in *C. gloeosporioides* and *C. siamense*. Typically, a conidium germinates to form a germ tube that extends and successively branches to establish the fungal colony. However, in certain conditions, the conidia and conidial germlings in close vicinity to each other may form a specialized hypha, called conidial anastomosis tubes (CATs) that connect conidia, resulting in an interconnected germling network (Read et al., 2010; Roca et al., 2005a, 2005b, 2003). Such network can act as a coordinated individual regulating its overall homeostasis by sharing nutrients, water, signal molecules and organelles, while allowing genetic exchange among conidia (Read et al., 2009). Since CAT fusion involves transfer of genetic material, it is thought that CAT fusion may account for HGT and/or HCT between otherwise incompatible strains (Ma et al., 2010; Mehrabi et al., 2011).

The CAT fusion has been demonstrated in many species of fungi (Roca et al. 2005b). However, the CAT fusion has been extensively studied in *C. lindemuthianum*, *C. gossypii*, *C. fructicola*, *C. nymphaeae*, *F. oxysporum*, *N. crassa* and *Venturia inaequalis* and for many of these fungi; *in-vitro* methods have been developed to study the biology of CAT fusion (Gonçalves et al., 2016; Kurian et al., 2018; Leu L.S., 1967; Roca et al., 2005a, 2003; Shahi et al., 2016; Wu et al., 2014). The CAT fusion has never been reported in *C. siamense*. Similarly, formation of multiple appressoria and CATs in *C. gloeosporioides* was reported on apple leaves (Araujo and Stadnik, 2013), however, there were no efforts undertaken to study the CAT fusion dynamics *in-vitro* and to understand the physiological requirements for CAT fusion in *C. gloeosporioides* and *C. siamense*. It is also not known, whether CAT fusion may contribute to generation of phenotypic and genetic diversity in these fungal pathogens.

The CAT fusion seems to involve a quorum sensing, wherein, the individual conidia senses their density by detecting the extracellular quorum sensing molecules secreted by their conidial population. There are indirect evidences that a diffusible chemo attractant is released during the CAT induction, which probably prime the conidia for CAT fusion (Roca et al., 2005a). The CAT inducer signal in *N. crassa* seems to activate a mitogen-activated protein (MAP) kinase, which is shown to be essential for CAT induction (Roca et al., 2005a). However, the chemical nature of quorum sensing molecule/inducer responsible for CAT fusion is not known till date in any fungi. It has been suggested that CAT fusion might play a vital role in life cycle of fungi by improving the chances of colony establishment during nutrient starvation (Roca et al., 2005b); however, there are contrasting reports on the relationship between CAT induction and nutrient availability e.g. CAT fusion in *C. lindemuthianum* is inhibited by nutrients and only occurs in water (Roca et al., 2003), while in *N. crassa* and *F. oxysporum*, CAT induction requires the presence of nutrients to occur (Kurian et al., 2018; Roca et al., 2005a). Moreover, whether any physiological stresses have any role to play in CAT fusion has also not been studied well till date.

Various inter-specific hybrids of filamentous plant pathogens have been described (Depotter et al., 2016) so far and the CAT fusion has been implicated for generation of fungal hybrids. In one such study, an interspecific hybrid of *C. lindemuthianum* and *C. gossypii* was reported to occur through CAT and the hybrids exhibited morphological and genetic properties of both the parent species (Roca et al., 2004). Though the existence of hybrids was predicted in *C. gloeosporioides* species complex (Cisar et al., 1994),
however, till date no study has demonstrated any inter-specific hybrids of *C. gloeosporioides* with any other species. Inter-specific hybridization or gene transfer between closely related species is a fast mean of genome evolution, which allows organisms to acquire novel traits to colonize new niches and/or novel hosts (Man et al. 2007; Bertier et al. 2013). Further, it is speculated that hybrid pathogens might have fitness advantages or disadvantages in a given niche.

In the present work, we aimed to study the physiological requirement and dynamics of CAT fusion between *C. gloeosporioides* and *C. siamense*. We also attempted to demonstrate that the CAT fusion is mediated through a quorum sensing like phenomenon in these fungi. Further we generated preliminary experimental proofs to support the role of CAT fusion in generating phenotypic and genotypic diversity in these fungi. The hybrid vigour of putative recombinants of *C. gloeosporioides* under stress conditions was also assessed.

**Materials And Methods**

1. **Fungal strains and species confirmation by ApMAT sequencing and phylogeny**

*Colletotrichum gloeosporioides* (CBS 953.97) and *Colletotrichum siamense* (NFCCI 3061) strains used in the study were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and National Fungal Culture Collection of India (NFCCI) (WDCM-932), Agharkar Research Institute, Pune, India respectively. The strains were maintained on PDA plates at 25°C and preserved in 15% glycerol at -80°C. The species identities of the isolates were initially confirmed by microscopic morphology and further the molecular identification of the above strains was done by *ApMAT* gene sequencing. Briefly, the genomic DNA (gDNA) was isolated from fungal colonies grown on Potato Dextrose Agar (Hi-Media Laboratories Pvt. Ltd.) plates for a week, by following a rapid DNA extraction protocol (Aamir et al., 2015) using FastPrep®24 tissue homogenizer (MP Biomedicals GmbH, Germany). The gDNA were subjected to polymerase chain reaction (PCR) amplification of the *ApMAT* gene using primers AMF - TCATTCTACGTATGTGCCCG and AMR - CCAGAAATACACCGAACTTGC using standard cycling conditions as described previously (Silva et al., 2012). The PCR products were purified using QIAquick PCR Purification Kit (QIAGEN), and sequenced using Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions on an ABI 3100 Avant Prism automated DNA sequencer (Applied Biosystems). The sequences generated in this study were deposited in NCBI-GenBank. The *ApMAT* region sequences of closely related species of *C. gloeosporioides* and *C. siamense* were retrieved from NCBI-GenBank and a phylogenetic tree was constructed using these sequences by following neighbor-joining method in MEGA7 to confirm the phylogenetic positions of *C. gloeosporioides* and *C. siamense* strains used in the present study.

2. **In-vitro conidial anastomosis tube (CAT) induction and their dynamics**

The *C. gloeosporioides* and *C. siamense* strains were inoculated on bean pod agar medium (autoclaved French bean pods submerged in 2% water agar) and were incubated in the dark at 25°C to induce
sporulation. Post-inoculation the conidia were harvested from these two species individually at different time points viz. 6, 10, 13, 17 and 20 days and suspended in distilled water. In order to induce CAT fusion, 0.2 ml conidial suspension (1×10^6 per ml) and 0.8ml distilled water were placed in the wells of a 24-well tissue culture plate (Tarsons, India) with the final concentration of conidia being 4×10^5/ml and incubated for 24 h, 48 h, 72 h and 96 h in the dark at 25°C. The CAT induction was carried out in three different ways; 1) CAT induction in only *C. gloeosporioides* conidia, 2) CAT induction in only *C. siamense* conidia, and 3) CAT induction in a co-culture of *C. gloeosporioides* and *C. siamense* conidia in equal numbers (Additional file 3: Figure S1). Different stages of CAT like induction, homing and fusion were examined at different time points using an Olympus BX53 DIC microscope equipped with Olympus DP73 camera and CellSens 1.13 imaging software. The CAT fusion was quantified as the percentage of conidia involved in fusion (Roca et al., 2003).

3. Effects of nutrients, physiological stresses and known CAT inducers on CAT induction

In order to study whether CAT fusion between *C. gloeosporioides* and *C. siamense* is dependent on nutrient availability and/or physiological stress conditions, the CAT fusion frequency was determined in the presence of different stresses and nutrients. The CAT fusion in 17 days old conidia of both the strains (co-culture) were induced in water, 100% Potato dextrose broth (PDB), 30mM H₂O₂ (oxidative stress), 1M NaCl and 1M Sorbitol (osmotic stress), at 40°C (heat stress) and 100µg/ml Copperoxychloride (antifungal stress). Further, the CAT fusion in 17 days old conidia of both the strains (co-culture) were also tested in the presence of various carbon and nitrogen compounds like 2% Glucose, 100mM KNO₃, combination of 2% glucose and 100mM KNO₃ (Shahi et al. 2016). The CAT induction was also assessed in the presence of previously known CAT inducers of other fungal systems e.g. 25mM NaNO₃, and 25mM MgCl₂ (Kurian et al. 2018). Effect of a known CAT inhibitor tryptophan (50 µM) (Fischer-Harman et al., 2012) on CAT induction in these fungi was also assessed. To study whether the CAT induction in these two species is mediated through MAP kinase kinase (MEK) pathways, the CAT fusion percentage was also determined in the presence of a MEK inhibitor InSolution™ PD98059 (5µM).

4. Detection of quorum sensing like phenomena during CAT fusion

Different numbers of conidia were tested to find out the threshold of conidial numbers required for inter-specific CAT fusion between *C. gloeosporioides* and *C. siamense*. The numbers of conidia tested were 4×10^2, 4×10^3, 4×10^4, 4×10^5 and 4×10^6 per ml of water. The conidial numbers represent sum of equal concentration of *C. gloeosporioides* and *C. siamense* conidia.

The basic chemical nature of unknown quorum sensing molecules secreted during CAT fusion was determined. Briefly, post inter-specific CAT fusion (96 hours of incubation) of the 17 days old conidia of both the species, the conidial suspension was collected from the tissue culture plate and conidia were separated from the liquid medium (water) by centrifugation at 10,000 rpm for 10 min at RT. The obtained CAT medium supernatant was filtered through Whatman® Syringe filter (pore size 0.45µm). This CAT medium supernatant (1 ml) was tested for their ability to induce CAT fusion in young (6 days) as well as
old conidia (17 days) of *C. gloeosporioides* and *C. siamense* in co-culture (4×10⁵ conidial concentration), wherein, the conidial co-culture in water (1 ml) was used as a control. These conidial co-cultures were incubated at 25°C for 72 h in the dark. The CAT induction ability of the CAT medium supernatant was tested even in the presence of nutrients *viz.* 100% PDB by inoculating 6 and 17 days old conidia of both the strains in (a) 1ml CAT medium supernatant, (b) 1ml 100% PDB individually and (c) combination of equal volume of CAT medium supernatant and 100% PDB (total volume 1ml).

To find out whether the putative quorum sensing molecule is lipid based or proteinaceous in nature, three different sets of experiments were performed *viz* 1) The CAT medium supernatant (1 ml) was treated with equal volume of chloroform, followed by centrifugation at 12000 rpm for 10 minutes at RT to obtain the aqueous CAT medium supernatant back. 2) The leftover chloroform (1ml) from the first set of experiment was treated with water (1ml) to re-extract the quorum sensing molecules, provided these molecules were lipid based in nature. 3) The CAT medium supernatant (1ml) was also treated with 4μl of proteinase K (10mg/ml) for 3 hours at 55°C. Such treated CAT medium supernatants were tested for their ability to induce CAT fusion in co-culture of 17 days old conidia of both the species.

5. **Nuclear and organelles transfer during CAT fusion**

To visualize the genetic transfer between *C. gloeosporioides* and *C. siamense* during CAT fusion, the DAPI staining was performed with some modifications (James et al., 1995; Roca et al., 2003). Briefly, 17 days old conidia of *C. gloeosporioides* and *C. siamense* were co-cultured in water for 72 h in 24 well tissue culture plates to induce CAT fusion as described above. To these wells, 20μl of sodium phosphate buffer (50μM), pH 7.0 and 10μl of DAPI solution (500μg/ml) was added. After about half an hour of incubation the DAPI solution was gently removed and the fused conidia were gently removed from the well and placed over the glass slide for fluorescence microscopy (ZIESS AXIO Imager.A2 microscope with AxioCam MRc5 camera). Few additional organelles staining experiments were performed using 100nM MitoRed (Sigma-Aldrich, USA), 100nM LysoTracker™ Red DND-99 (Thermo Fisher Scientific), and 100nM Nile Red (Sigma-Aldrich, USA)(Hickey et al., 2004; Meadows BRA, 2012; Yin et al., 2010) to visualize the transfer of mitochondria, lysosomes and lipid droplets, respectively during the CAT fusion. Confocal microscopy was performed to visualize the migration of above-mentioned organelles using Leica SP8 confocal microscope and images were processed and analyzed by LAS X software. The excitation and emission spectra used for MitoRed, LysoTracker™ Red DND-99 and Nile Red staining were 569/594, 577/590 and 520/620, respectively.

6. **Assessment of colony morphology of progenies generated through intra and inter-specific CAT fusion**

The colony morphologies of putative recombinant conidia generated post intra-specific (self-fusion) and inter-specific CAT fusions were studied by harvesting them by following a single spore isolation method (Ho and Ko, 1997). Briefly, intra-specific and inter-specific CAT fusions in and between *C. gloeosporioides* and *C. siamense* were carried out as described in above mentioned method. The conidia were harvested
from the 24 well tissue culture plate post CAT fusion (after 96 hours of incubation) by centrifugation and the concentration was adjusted to approximately 1 conidia/µl by diluting the conidia with sterile water. About 50 circles (3mm diam.) were drawn on the bottom of water agar plates. A 1µl drop of conidial suspension was placed on the water agar surface above each circle. After incubation at 25°C for 12-24 h, each circle was inspected microscopically at 100X magnification from the bottom of the plate. The circle containing single germinating conidium was marked and conidia in those circles were individually transferred to PDA plates. The resulting pure colonies originating from single spore isolation were termed as putative recombinants. The colony morphologies of these putative recombinants were analyzed. In cases of inter-specific CAT fusion, the genetic backgrounds of the putative recombinants were checked by ApMAT gene sequencing so as to find out whether a given putative recombinant belongs to \( C. \) gloeosporioides or \( C. \) siamense. The ApMAT gene sequences of the putative recombinants generated in this study were deposited in NCBI-GenBank. In order to rule out the possibility of any other mechanisms (e.g. spontaneous phenotypic heterogeneity or stress induced genomic alterations) for generation of phenotypic diversity, the single conidium of each species were harvested from the 17 days old cultures of these two fungi, grown separately and without subjecting them to CAT fusion. These vegetatively grown conidia were allowed to develop as a colony and their morphological characteristics were recorded.

7. Amplified fragment length polymorphism (AFLP) analysis of \( C. \) gloeosporioides and \( C. \) siamense parental strains and their putative recombinants

In order to determine genetic diversity in putative recombinants of \( C. \) gloeosporioides and \( C. \) siamense, an AFLP assay was performed for both the parental strains [\( C. \) gloeosporioides parent (CGP) and \( C. \) siamense parent (CSP)] and their 20 recombinants [\( C. \) gloeosporioides recombinants (CG1-10) and \( C. \) siamense recombinants (CS1-10)] generated post-CAT fusion. Approximately 50ng of genomic DNA of each of the strain was subjected to a combined restriction-ligation procedure with a mixture containing 50 pmol of the \( HpyCH4iV \) adapter, 50 pmol of the \( MseI \) adapter, 2U of \( HpyCH4iV \) (New England Biolabs, Ipswich, MA), 2U of \( MseI \) (NEB), and 1U of T4 DNA ligase (NEB) and 0.2µl of 100x Bovine Serum Albumin (BSA) in a total volume of 20 µl of 1X reaction for 2 h at 37°C then 1 h at 16°C in Applied Biosystems ProFlex PCR System. Adapters were made by mixing equimolar amounts of complementary oligonucleotides (5'-CTCGTAGACTGCAGTC-3' and 5'-CGGGTACGCAGTC-3' for \( HpyCH4iV \); 5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3' for \( MseI \)) and heating them to 95°C for 2 min, with subsequent slow cooling to ambient temperature in Applied Biosystems ProFlex PCR System. One microliter of the 10 times diluted restriction-ligation mixture was used for pre-selective amplification in a volume of 25 µl under the following conditions: 1µM \( HpyCH4iN \) forward primer (5'-GTAGACTGCAGTCCTGAC-3'), 1µM \( MseI \) reverse prime (5'-GATGAGTCCTGACTAATGAG-3'), 0.2mM each deoxynucleoside triphosphate (dNTP), and 1U of \( Taq \) DNA polymerase (Sigma Aldrich, USA) in 10X reaction buffer containing MgCl2. Amplification was performed as follows: an initial denaturation step for 5 min at 94°C, 20 cycles of 30s of denaturation at 94°C and 30s of annealing at 56°C and 2 min of extension at 72°C. These pre selective PCR products (1µl) were used as template for selective amplification in a volume of 25 µl under the following conditions: 1µM \( HpyCH4iV \) forward primer, 1µM selective \( MseI \) reverse primer with underlined selective base pairs (5'-GATGAGTCCTGACTAATGAG-AC-3'),
0.2mM each deoxynucleoside triphosphate (dNTP), and 1U of *Taq* DNA polymerase (Sigma Aldrich, USA) in 10X reaction buffer containing MgCl₂. Selective amplification was performed as follows: an initial denaturation step for 5 min at 94°C, 20 cycles of 30s of denaturation at 94°C and 30s of annealing at 66°C and 2 min of extension at 72°C. Cycling was then continued for a further 30 cycles, with an annealing temperature of 56°C and a final extension at 72°C for 10 min and hold at 4°C (Chakrabarti et al., 2010). The selective PCR products were run on native polyacrylamide gel electrophoresis (PAGE) to determine the banding pattern of parents and their recombinants. For PAGE, 100ml (5%) gel solution was prepared by adding 12.5ml of 40% acrylamide and bis-acrylamide solution (19:1), 10ml 10x Tris-Borate EDTA, 50mg Ammonium persulfate (APS) and 40µl Tetramethylethylenediamine (TEMED). Eight µl of selective amplified product and 100bp DNA marker were loaded in the native PAGE gel and it was run on C-DASG-400-50 Dual Adjustable Mega-Gel Electrophoresis System (CBS Scientific) at 200 Volts for 3-4 hours. After the electrophoresis, the gel was stained with Silver Nitrate stain (Elouafi and Nachit, 2004). The stained gel was observed under white light and photographed with Canon EOS 600D.

8. Assessment of hybrid vigour of putative recombinants under stress conditions

The fitness of parent strains and putative recombinants of *C. gloeosporioides* was tested under different stresses viz. oxidative stress (H₂O₂), osmotic stresses (NaCl and Sorbitol) by employing surface plate assay (Zafra et al., 2015). Briefly, the *C. gloeosporioides* putative recombinants and parent strain (1x10⁴ conidia) were individually inoculated on the centre of PDA plates containing 30mM H₂O₂, 1M NaCl and 1M Sorbitol separately. Plates were incubated at 25°C for 7 days and mycelium radial extension rate measurements (cm d⁻¹) were made every 24 h manually with help of ruler. Fungal growth assays were structured by using an 11x3 factorial design (11 strains and 3 different stress mediums).

9. Statistical analysis

All data were analyzed by Analysis of Variance (ANOVA) with GraphPad Prism 5 Statistics Software. Differences with a *p value* < 0.05 were considered statistically significant. All assays were performed in triplicates and with 300 conidial numbers, wherever applicable.

Results

High frequency of inter-specific versus intra-specific CAT fusion in *C. gloeosporioides* and *C. siamense*

*Colletotrichum gloeosporioides* CBS 953.97 could be differentiated from *C. siamense* NFCCI 3061 by having cylindrical conidia while the later had fusiform shaped conidia (Fig. 2D-E) (Prihastuti et al., 2009). The BLASTn analysis of *ApMAT* DNA sequence of these fungal strains showed 100% sequence similarity with *C. gloeosporioides* and *C. siamense* (Additional file 1: Table S1). Further, the *ApMAT* based phylogenetic analysis revealed that these two species of *Colletotrichum* clustered in their respective clades in phylogenetic tree, thereby confirming their identity and phylogenetic positions (Fig. 1).
When different aged conidia of *C. gloeosporioides* and *C. siamense* were analyzed individually for CAT fusion, it was observed that the CAT fusion occurred in very low frequency in 6 d old conidia and thereafter percentage of CAT fusion increased with the increasing age of conidia. The CAT fusion frequency reaches to its peak when the conidial age was 17 days, thereafter it starts decreasing (Fig. 2A). It was observed that when different aged conidia were harvested and incubated in distilled water for 24, 48, 72 and 96 h, the maximum CAT fusion frequency was observed at 72 h post-incubation (Fig. 2B). The CAT fusion percentage observed in *C. gloeosporioides* and *C. siamense* individually (intra-specific) were 11% and 12%, respectively (Fig. 2C). However, when 17 days old conidia of these two different species were co-cultured in distilled water for CAT induction (for 72 h), interestingly the inter-specific CAT fusion percentage observed was as high as 25%, which was significantly higher than intra-specific CAT fusion percentage (Fig. 2C). The representative microscopic images of intra-specific and inter-specific CAT fusion in/between *C. gloeosporioides* and *C. siamense* have been depicted in figure 2 D-F. Since, the highest CAT fusion percentage was observed in co-culture of 17 days old conidia of *C. gloeosporioides* and *C. siamense* and 72 h incubation time in water for CAT induction *in-vitro*, most of the further experiments were conducted using these optimized parameters and co-culture of these two different species.

Different stages of the inter-specific CAT fusion between *C. gloeosporioides* and *C. siamense* were studied (Fig. 3). Initially conidia get attracted and come closer to each other due to the effect of extracellular secretory molecules constituting the first stage of CAT fusion known as CAT induction (Fig. 3A). Subsequently, conidia with CATs home towards each other (Fig. 3B) and finally they fused to each other (Fig. 3C). Later on, with increasing incubation time CAT connections expanded and formed CAT pre-network (Fig. 3D) and the final CAT network of inter-connected conidia (Fig. 3E-F) till 72 h of incubation. After 72 h CATs started to detach and by 96 h, conidia connected through CATs completely detached and got separated from each other (Fig. 3G).

2. **CAT fusion is dependent on nutrient availability and osmotic stress**

The conidia of *C. gloeosporioides* and *C. siamense* failed to undergo CAT fusion in the presence of nutrient rich medium like 100% PDB (Fig. 4). As shown previously, the high percentage of CAT fusion (22.3%) was observed in water only. However, when the conidia were incubated in water with glucose, KNO₃ and combination of glucose and KNO₃, the CAT fusion frequency decreased to 4%, 12% and 2%, respectively (Fig. 4). Among different stress conditions, osmotic (1M NaCl and 1M sorbitol) and oxidative stresses (30mM H₂O₂) could induce the CAT fusion in these fungi (Fig. 4). The maximum CAT fusion (25.7%) was detected under osmotic stress exerted by 1M sorbitol (Fig. 4). The inter-specific CAT fusion was also observed in the presence of other known CAT inducers like 25mM NaNO₃, and 25mM MgCl₂ at the tune of 12%, and 12.8% respectively, however, the percentage CAT fusion was lower than water only (Fig. 4). Presence of tryptophan could reduce the CAT fusion to 5.9%. The conidia failed to induce CAT fusion in the presence of InSolution™ PD 98059, an inhibitor of MAP kinase kinase, thereby suggesting the involvement of MAPK pathway in inter-specific CAT fusion between *C. gloeosporioides* and *C. siamense* (Fig. 4).
3. **Inter-specific CAT fusion is mediated through quorum sensing like phenomena**

A density of $4 \times 10^4$ and $4 \times 10^5$ conidia per milliliter (ml) of water was found to be optimal for inter-specific CAT fusion (Fig. 5A), while the maximum CAT fusion % was seen in $4 \times 10^5$ conidia per ml. When inter-specific CAT fusion in young (6 days) and old (17 days) conidia of both the species were assessed in water, the younger conidia showed low levels of CAT fusion (11%) as compared to the older conidia (23%). While in the presence of 100% PDB, conidia of both the age showed negligible CAT fusion, thereby suggesting that rich nutrients do not support CAT fusion. Interestingly, increase in CAT fusion percentage was seen in the presence of CAT medium supernatant (14% in 6 days and 27% in 17 days old conidia), suggesting that the quorum sensing molecules present in CAT medium supernatant could even induce CAT fusion in younger conidia. Significant increase in percentage CAT fusion was seen in both young (8%) and old conidia (14%) even in 100% PDB, when the CAT medium supernatant was supplemented with it (Fig. 5B). The proteinase-k treated CAT medium supernatant could induce inter-specific CAT fusion (18%), while chloroform treated CAT medium supernatant failed to induce CAT fusion in these fungi, suggesting that the putative quorum sensing molecule or CAT inducer was probably a lipid-based molecule (Fig. 5C). To reconfirm the lipid nature of CAT inducer, the leftover chloroform from chloroform treatment of CAT medium supernatant was re-extracted with water and interestingly such water re-extract could induce significantly high CAT fusion percentage (up to 40%), and thereby suggesting that the CAT inducer might be a lipid-based molecule (Fig. 5C). These results suggest that CAT fusion was dependent on conidial threshold and putative extracellular quorum sensing molecules, which are hallmark of quorum sensing like phenomenon.

4. **Movement of nuclei and other cell organelles during CAT fusion**

DAPI staining showed that the conidia of both the species, which are committed for CAT fusion were essentially bi-nucleated, however, the number of nuclei increased up to 3-4 in the beginning of nuclear transfer between the conidia. Out of such 3-4 nuclei, one nucleus/part of nucleus was transferred through CATs between conidia of these two species (Fig. 6). Mito Red staining revealed the movement of mitochondria through fused CATs between conidia of *C. gloeosporioides* and *C. siamense* (Fig. 7A-C). Mitochondria's were abundantly present in both the conidia and CATs suggesting that the CAT fusion is an energy intensive process. The transfer of lysosomes and lipid droplets through CATs was also evident by LysoTracker™ Red DND-99 (Fig. 7D-F) and Nile Red staining (Fig. 7G-I), respectively.

5. **Inter-specific CAT fusion generated phenotypic and genotypic diversity**

The putative recombinants generated through inter-specific CAT fusion between *C. gloeosporioides* and *C. siamense* showed phenotypic variations in colony characteristics as compared to their parent strains (Fig. 8). We selected 10 such putative recombinants of *C. gloeosporioides* (CG1 to CG10) and *C. siamense* (CS1 to CS10) species background (generated through inter-specific CAT fusion) with some phenotypic variations in their colony morphology (Fig. 8). Among both the species background, a greater number of *C. gloeosporioides* putative recombinants showed significant phenotypic variations as
compare to *C. siamense* putative recombinants (Additional file 2: Table S2). Microscopic analysis of the parent strains and the putative recombinants of *C. gloeosporioides* and *C. siamense* revealed that the recombinants were pure culture only. The *ApMAT* gene sequencing of these putative recombinants accurately identified their background species, ruling out the possibility of mixed conidia of these two species. The *ApMAT* gene sequences of recombinants generated in this study were deposited in GenBank with accession numbers listed in Additional file 1: Table S1.

Possibility of any other mechanisms for generation of phenotypic diversity e.g. intra-specific CAT fusion (or self-CAT fusion), spontaneous phenotypic heterogeneity or stress induced genomic alterations were ruled out, because progenies generated through intra-specific CAT fusion (or self-CAT fusion) in *C. gloeosporioides* and *C. siamense* individually did not show any apparent phenotypic variations in colony morphology (Additional file 4: Fig. S2A). Further, the single conidia (17 days old) of each species when grown vegetatively as colony (without CAT fusion), they did not show any phenotypic variations, thereby suggesting that the phenotypic variations obtained were due to a possible transfer of genetic material through inter-specific CAT fusion only (Additional file 4: Fig. S2B).

The AFLP banding pattern of some recombinants of *C. gloeosporioides* and *C. siamense* were different compared to their parent strains. We observed significantly more variations in AFLP banding pattern in *C. gloeosporioides* recombinants namely CG1, 3, 8 and 9 as compared to *C. gloeosporioides* parent strain (Fig. 9A). On the other hand, very fewer variations were observed in *C. siamense* recombinants banding pattern (only in CS1) as compared to *C. siamense* parent strain (Fig. 9B). The presence of extra band was indicated as an asterisk just beneath them in Figure 9. Interestingly, the extra bands observed in *C. gloeosporioides* recombinants CG1, 3, 8 and 9 was not present in *C. gloeosporioides* parent strain but present in *C. siamense* parent strain (Fig. 9A), which denotes that these extra bands in *C. gloeosporioides* recombinants might have got transferred from *C. siamense* through CAT fusion, thereby suggesting an inter-specific genetic transfer.

6. **Putative recombinants showed varied fitness under stress conditions**

Out of 10 *C. gloeosporioides* putative recombinant strains, 7 recombinants showed significant varied growth rate under oxidative stress, out of which three recombinant strains showed higher growth rate as compared to the parent strain. On the contrary, 4 out of 7 recombinant strains showed reduced growth rate under oxidative stress (Fig. 10A). With respect to osmotic stress (1M NaCl), only 1 out of 10 putative recombinant strains showed higher growth rate, and 7 showed reduced growth rate as compared to the parent strain (Fig. 10B). Further under sorbitol mediated osmotic stress, 3 out of 10 putative recombinant strains showed higher growth rate, and 4 showed reduced growth rate as compared to the parent strain (Fig. 10C).

**Discussion**

The studied fungal species *C. gloeosporioides* and *C. siamense* have previously been shown to be different valid species, however, the *ApMAT* based phylogenetic analysis has shown that *C.*
gloeosporioides was more closely related to C. siamense then C. asianum and C. fructicola. (Silva et al., 2012; Sharma et al., 2013). Our strains belonging to these two species were also rightly placed in the phylogenetic tree (Fig. 1). We have optimized the *in-vitro* conditions for CAT induction in *C. gloeosporioides* and *C. siamense*. We observed significant CAT induction in 17 days old conidia of both these species individually (Fig. 2A). Among different species of *Colletotrichum*, it was reported that 16 days old culture of *C. lindemuthianum* and *C. gossypii* undergo CAT fusion, while 20 days old culture of *C. fructicola* and *C. nymphaeae* formed CATs (Gonçalves et al., 2016; Roca et al., 2004, 2003). If CAT fusion in other fungal genera is considered, the conidial age requirements for *Fusarium oxysporum* and *Neurospora crassa* were 7–10 and 4–5 days, respectively (Kurian et al., 2018; Roca et al., 2005a; Shahi et al., 2016). Our result seems to fit with the conidial age requirement (older conidia) of the genus *Colletotrichum* for CAT induction. The CAT fusion percentage was about 11% for both *C. gloeosporioides* and *C. siamense* individually (Fig. 2C). However, the CAT fusion frequency was significantly high (25%) when these two species were subjected to CAT induction in the same well, suggesting that the inter-specific CAT fusion was preferred as compare to intra-specific CAT fusion in these two fungal species (Fig. 2C). Inter-specific CAT fusion has also been demonstrated between *C. lindemuthianum* (harboring hygromycin resistance) and *C. gossypii*, however, the frequency of these inter-specific fusions was very low even on strong hygromycin selection (Roca et al., 2004). Interestingly, in our experiments, there was no selection pressure for inter-specific CAT fusion between *C. gloeosporioides* and *C. siamense*, still the CAT fusion with the other species was preferred. It is hypothesized that inter-specific CAT fusion provides a unique opportunity to exchange the gene pool between two different species, which would possibly generate more genetic diversity as compared to intra-specific CAT fusion. It has been shown previously that the vegetative incompatibility response is suppressed during colony initiation in *C. lindemuthianum* during CAT fusion (Ishikawa et al., 2012). Thus, by removing the vegetative incompatibility barrier, CAT fusion may allow asexual fungi to increase their genetic diversity. The CATs have been divided into three phases; CAT induction, CAT homing and CAT fusion (Roca et al., 2005b). These phases were also observed in inter-specific CAT fusion between *C. gloeosporioides* and *C. siamense* (Fig. 3).

While studying the effect of availability of nutrients on CAT fusion frequency, we observed that the conidia (6 and 17 days old) of *C. gloeosporioides* and *C. siamense* failed to undergo CAT fusion in the presence of nutrient rich medium like 100% PDB (Fig. 4). As shown previously, the high percentage of CAT fusion was observed in water only (Ishikawa et al., 2010). The availability of glucose (carbon source), KNO₃ (nitrogen source), and combination of glucose and KNO₃, further lessen the CAT fusion frequency as compared to water only (Fig. 4). These observations suggest that the CAT fusion in *C. gloeosporioides* and *C. siamense* occur only in nutrient limited conditions. Our observation corroborates with the previous findings in several ascomycetes’ fungi, wherein, the CAT fusion was inhibited in the presence nutrients or rich organic growth medium (Roca et al. 2005a; Shahi et al. 2016; Kurian et al. 2018). The known CAT inducers of *Fusarium sp*. e.g. NaNO₃, and MgCl₂ could induce some level of CAT fusion in *C. gloeosporioides* and *C. siamense*, however, the CAT fusion frequency was significantly less as compared to water suggesting that the physiological requirement for CAT induction in different fungi may not be
very identical. Tryptophan, a known CAT fusion inhibitor also reduced the inter-specific CAT fusion frequency in these fungi up to some extent (Fig. 4) (Fischer-Harman et al., 2012).

A correlation has been established between some important developmental processes like sporulation and biofilm formation to the environmental stresses in few ascomycetous fungal species (Emri et al., 2015; Jaimes-Arroyo et al., 2015; Linz et al., 2013; Orosz et al., 2018; Reverberi et al., 2010; Zheng et al., 2015). Therefore, we further assessed whether the inter-specific CAT fusion between C. gloeosporioides and C. siamense is also dependent on physiological stresses like oxidative, hyperosmotic, heat and antifungal stresses. We observed that the CAT fusion frequency was much lower under heat and antifungal stresses, however, the CAT fusion was found to be higher in oxidative and osmotic stresses in (Fig. 4). Even the hyperosmotic stress exerted by sorbitol induced much higher CAT fusion percentage as compared to water (Fig. 4). The possible reason for relatively high frequency CAT fusion by hyperosmotic and oxidative stresses may be because these stresses activate the mitogen-activated protein kinase signaling pathway, which was shown to be essential for CAT fusion in N. crassa (Roca et al., 2005a). We have also shown that the inter-specific CAT fusion in C. gloeosporioides and C. siamense gets inhibited by a MAP kinase inhibitor InSolution PD 98059 (Fig. 4), thereby suggesting a possible role of MAP kinase pathway in CAT fusion in these fungi. Therefore, we hypothesize that a potential cross-talking among MAPK cascades during hyperosmotic and oxidative stresses, in turn induce CAT fusion fortuitously, however, further studies are needed in this direction.

In C. lindemuthianum, C. gossypii, C. fructicola, C. nymphaeae, Fusarium oxysporum, Neurospora crassa and Venturia inaequalis, the CAT induction is dependent on conidial density, thereby suggesting the involvement of an extracellular CAT inducer. In all these fungi, the conidial number threshold required for CAT induction is more or less same i.e. 1 × 10⁶ conidia/ml (Gonçalves et al., 2016; Kurian et al., 2018; Leu L.S., 1967; Roca et al., 2005a, 2003; Shahi et al., 2016). The combined conidial number requirement for inter-specific CAT fusion in C. gloeosporioides and C. siamense was found to be 4 × 10⁵ conidia/ml (2 × 10⁵ conidia/ml for each species). It suggests that for an efficient CAT fusion, a threshold conidial number is required and any concentration lesser then that would not result in efficient CAT fusion (Fig. 5A). The CAT induction was shown to be mediated through release of extracellular quorum sensing molecule/s, however, the chemical identity of such molecule/s is not known till date in any fungi known to undergo CAT fusion (Roca et al., 2005a). We have shown that the young conidia were not optimal for CAT fusion, however, when the young conidia were incubated in filtered CAT medium supernatant of older conidia (17 days), they could also undergo CAT fusion up to some extent, thereby suggesting that some molecule/s are secreted in the medium, which could induce CAT fusion even in the younger conidia (Fig. 5B). We have also demonstrated that availability of nutrients (100% PDB) inhibited CAT fusion in C. gloeosporioides and C. siamense, however, when the conidia of these fungi were incubated in 100% PDB supplemented with CAT medium supernatant, then the conidia of both the age could undergo CAT fusion, thereby, suggesting that though the nutrients (100% PDB) had the potential to inhibit the CAT fusion but the quorum sensing molecules could still induce the CAT fusion in the presence of nutrients (Fig. 5B). It was suggested that the CAT inducer might be of peptide or proteinaceous in nature, which gives species
specificity for CAT fusion, thereby preventing non self-fusion between species (Roca et al., 2005a). We have also attempted to determine the basic chemical nature of the quorum sensing molecule/s involved in CAT fusion between *C. gloeosporioides* and *C. siamense*, by treating the CAT medium supernatant with chloroform and proteinase K separately. We found that proteinase K treated supernatant could retain the CAT inducing ability, thereby suggesting that the potential quorum sensing molecule was not a peptide or protein. However, the chloroform extraction abolishes the CAT induction ability of the CAT medium supernatant, suggesting that the potential CAT inducer might be a lipid-based molecule (Fig. 5C). Further, we have also shown that when leftover chloroform from chloroform treatment of CAT medium supernatant was re-extracted with water and such re-extract could induce significantly high CAT fusion percentage (up to 40%) in older (17 days) conidia suggesting that the CAT inducer is a lipid-based molecule (Fig. 5C). The high CAT fusion percentage seen in re-extract could be because the quorum sensing molecule got purified from other impurities, thereby increased the CAT fusion percentage. We hypothesize that the QS molecules involved in CAT fusion between *C. gloeosporioides* and *C. siamense* is probably a lipid-based molecule and we are currently trying to decipher the chemical structure and identity of this QS molecule.

Previous studies have indicated that CAT fusion may facilitate horizontal gene/chromosome transferred. Nuclei were shown to move between fused conidia of many fungi *viz.* *C. lindemuthianum*, *C. gossypii*, *C. nymphaeae*, *F. oxysporum*, and *N. crassa* (Gonçalves et al., 2016; Kurian et al., 2018; Roca et al., 2005a, 2003; Shahi et al., 2016). By performing DAPI staining, we also observed the movement of nuclei during CAT fusion between *C. gloeosporioides* and *C. siamense* (Fig. 6). This nuclear transfer between the two species signifies that this inter-specific CAT fusion is not accidental; indeed, it does involve exchange of nuclear material. We have also shown movement of other cell organelles *e.g.* mitochondria, lysosomes and lipid droplets during the CAT fusion, which signifies that there has been an active exchange of cellular content between the two species (Fig. 7). These results are first ever evidence of cell organelles transfer during the CAT fusion in any *Colletotrichum* species so far. Such organelle transfer during CAT fusion has been reported for *F. oxysporum*, wherein, the movement of mitochondria, vacuoles and lipid droplets was documented (Kurian et al., 2018; Ruiz-Roldán et al., 2010). Nuclear transfer through CAT fusion could be a form of horizontal gene transfer, which might generate genetic and phenotypic diversity and confer selective advantage to survive under the nutrient limitation and other stress conditions. It has been reported that the inter-specific CAT fusion between *C. lindemuthianum* and *C. gossypii*, resulted some hybrid colonies, which exhibited morphological variation with distinct phenotypic sectors of both parental types (Roca et al., 2004). Occurrence of heterokaryotic sectored colonies post CAT fusion has also been demonstrated in *C. lindemuthianum* (Ishikawa et al., 2012). In order to understand the role of inter-specific CAT fusion in generation of genetic and phenotypic diversity in *C. gloeosporioides* and *C. siamense*, we have carried out three experiments: (1) Post-CAT fusion between *C. gloeosporioides* and *C. siamense*, the conidia were harvested and grown as a single colony in separate Petri plates, wherein, each colony originated from a single conidium (in which the genetic transfer might have happened). The resulting colonies have shown significant phenotypic variations in colony characteristics (Fig. 8). By observing the colony characteristics, we could not identify that the colony developed originally from
which fungal conidia (\textit{C. gloeosporioides} or \textit{C. siamense}); moreover, one might argue that whether these phenotypic variations are bona fide variations or it is a result of mixing of conidia of two different species, though we strictly followed single spore isolation protocol. These two species can be differentiated based on their conidial morphologies, wherein, \textit{C. gloeosporioides} conidia are cylindrical, while \textit{C. siamense} are fusiform in shape (Prihastuti et al., 2009). Therefore, by doing light microscopic studies on these putative recombinant colonies, we could tentatively establish the species background of these recombinants, however, at times it has been difficult to differentiate the post-CAT fusion generated conidia based on the morphology, therefore, the \textit{ApMAT} gene sequencing accurately confirmed the species background of these putative recombinants (Additional file 1: Table S1). The \textit{ApMAT} gene sequencing proved that whatever putative recombinants were observed, they were indeed bona fide recombinants of \textit{C. gloeosporioides} and \textit{C. siamense}. Among both the species, a greater number of \textit{C. gloeosporioides} putative recombinants showed significant phenotypic variations as compare to \textit{C. siamense} putative recombinants (Additional file 2: Table S2). (2) In order to prove that the phenotypic variations observed in putative recombinants were genuinely generated due to inter-specific CAT fusion between these two species, the results of our control experiment showed that intra-specific CAT fusion (self-fusion) in \textit{C. gloeosporioides} and \textit{C. siamense} individually did not generate apparent colony morphology variations in their progenies (Additional file 4: Fig. S2A). (3) There can be another argument saying that CAT fusion might not be a reason for the observed phenotypic variations in the \textit{C. gloeosporioides} and \textit{C. siamense} colonies (which developed from single conidium post CAT fusion) and such variation could have generated spontaneously as a result of phenotypic heterogeneity exhibited by many fungi or stress induced genomic alterations. Phenotypic heterogeneity’ refers to a phenomenon whereby individual cells within clonally-derived populations, that have a uniform genetic background, can nevertheless display differences in phenotype (Hewitt et al., 2016). It has also been seen that prolonged starvation/stress could cause genomic rearrangements and transposon movement in fungi, thereby generating phenotypic variations (Coyle and Kroll, 2008; Miousse et al., 2015). To rule out these two possibilities, when \textit{C. gloeosporioides} and \textit{C. siamense} were grown for 17 days, it caused nutrient limitation stress and such conidia when developed as colonies did not show any significant phenotypic variation with respect to colony characteristics as compared to their parental strains (Additional file 4: Fig. S2B). Therefore, the above-mentioned 2nd and 3rd experiment further confirms that the phenotypic variation (colony characteristics) generated in putative recombinant was not spontaneous and was due to inter-specific CAT fusion only.

In order to further explore that the CAT fusion between these two species indeed involved genetic exchange and responsible for genetic variations, we performed AFLP of genomic DNA isolated from \textit{C. gloeosporioides} and \textit{C. siamense} parents’ strains, and their putative recombinants (Fig. 9). Interestingly, we observed a much variable AFLP banding pattern in \textit{C. gloeosporioides} putative recombinants as compared to \textit{C. gloeosporioides} parent strain. Interestingly, we also observed that few \textit{C. gloeosporioides} recombinants showed presence of some extra bands, which are present in \textit{C. siamense} and not in \textit{C. gloeosporioides} parent strains. This suggest that during CAT fusion, some amount of DNA might have got transferred between the two species, which resulted in variable AFLP banding pattern in \textit{C.
gloeosporioides putative recombinants. These observed genotypic variations in C. gloeosporioides putative recombinants might be responsible for the above-mentioned phenotypic variations in C. gloeosporioides putative recombinants. Further, we do not see a significant variation in AFLP banding pattern in C. siamense putative recombinants, which interestingly corroborates with our phenotypic variation data, wherein, we did not see a significant phenotypic variation in the colonies of C. siamense putative recombinants as well. These variable AFLP patterns were reproducible thereby ruling out the possibility of any artifact or procedural borne variation in AFLP banding pattern. Our results suggest that the inter-specific CAT fusion between C. gloeosporioides and C. siamense, involved some level of genetic exchange, which resulted in phenotypic variations in C. gloeosporioides putative recombinants. However, further experiments are warranted to confirm the same.

We have shown that a greater number of C. gloeosporioides recombinants exhibited phenotypic variations (Fig. 8); therefore, we tested the fitness of these recombinants only. We observed that there were few recombinants (e.g. CG1 and CG4) showed growth advantages under certain stress conditions (oxidative stress) as compared to others and the same recombinants showed reduced growth under a different stress condition (osmotic stress). One putative C. gloeosporioides recombinant CG2 exhibited higher growth rates under oxidative as well as osmotic stress as compared to the parent strain of C. gloeosporioides (Fig. 10). The observed growth advantages might be due to heterosis, wherein, recombinants were more heterozygous than their parents.

This is the first study to determine the optimal conidial density, conidial age, nutritional factors, and other physiological requirements for CAT induction between C. gloeosporioides and C. siamense. We discovered a significantly efficient inter-specific CAT fusion between these two fungi under no selection pressure, which is very intriguing. We have also generated some preliminary experimental proofs to link the role of inter-specific CAT fusion in generation of phenotypic and genotypic diversity in these fungi. Further, the putative recombinants generated post-CAT fusion was also followed up for their fitness in different stress conditions and it was seen that CAT fusion might be responsible for generation of the offspring’s which may have growth advantages in a given niche. Therefore, to summarize, the observed colony phenotypic variations, AFLP band polymorphism and varied fitness of the putative recombinants together suggests a possible genetic rearrangement through inter-specific CAT fusion. The present study will help to understand the genetic exchange/transfer mechanisms and generation of inter-specific hybrids or recombinants in C. gloeosporioides and C. siamense in future.

Description Of Supplementary Material

Additional file 1: Table S1: Fungal cultures, their ApMAT gene-based identification and GenBank accession numbers.

Additional file 2: Table S2: Percentage phenotypic variations of C. gloeosporioides and C. siamense colonies generated post inter-specific CAT fusion.
Additional file 3: Figure S1: A schematic representation of *in-vitro* CAT induction protocol. Cg: *C. gloeosporioides* and Cs: *C. siamense*.

Additional file 4: Figure S2: Colony morphologies of *C. siamense* and *C. gloeosporioides* parent strains and their progenies generated post intra-specific CAT fusion and vegetative growth. a Colony morphologies of parent *C. siamense* and *C. gloeosporioides* strains and their progenies (CSA-CSJ and CGA-CGJ) derived post intra-specific CAT fusion individually (self-fusion) by single spore isolation. b Colony morphologies of parent *C. siamense* and *C. gloeosporioides* strains and their progenies (CSV1-CSV10 and CGV1-CGV10) obtained from vegetatively grown *C. gloeosporioides* and *C. siamense* strains (17 days old), individually without CAT fusion. Scale Bar = 20 µm.

**Declarations**

**Acknowledgement**

We are thankful to the Director, MACS’ Agharkar Research Institute for providing the necessary facility to carry out the research work. AB is thankful to SERB-DST for sanctioning the core research grant (CRG/2018/001786). NM acknowledges Council for Scientific and Industrial Research (CSIR) New Delhi for the junior research fellowship. We are grateful to Dr. Ravindra Patil from Agharkar Research Institute, Pune for providing us the native PAGE facility.

**Adherence to national and international regulations**

*Colletotrichum gloeosporioides* (CBS 953.97) and *Colletotrichum siamense* (NFCCI 3061) strains used in the study were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and National Fungal Culture Collection of India (NFCCI) (WDCM-932), Agharkar Research Institute, Pune, India respectively.

**Authors’ contributions**

NM and AB designed the research, NM performed the experiments, NM and AB analyzed and interpreted the experiments and results. They also wrote the manuscript; all authors read, revised and approved the manuscript.

**Funding**

This study was funded by Science and Engineering Research Board (SERB) – Department of Science and Technology, New Delhi, India. The project grant number is CRG/2018/001786.

**Availability of data and materials**

ApMAT DNA Sequences of *C. gloeosporioides* and *C. siamense* parent and their recombinant strains were deposited to NCBI and accession numbers listed in Additional file 1: Table S1.
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declared that they have no competing interests.

References


Manners JM, He C (2011) Slow-growing heterokaryons as potential intermediates in supernumerary chromosome transfer between biotypes of Colletotrichum gloeosporioides. Mycol Prog 10:383–388

Meadows BRA (2012) Live cell imaging of lipid droplet distribution and motility in the filamentous fungus Ustilago maydis. The University of Exeter


Figures
Figure 1

ApMAT gene-based phylogenetic placement of C. gloeosporioides (CBS 953.97) and C. siamense (NFCCI 3061) with the closely related species. The tree was constructed using the neighbor joining method in MEGA 7. The scale bar indicates the number of expected substitutions per site. The numbers provided on branches are frequencies with which a given branch appeared in 1000 bootstrap replications. The tree was rooted with C. xanthorrhoeae.
Figure 2

Intra and inter-specific CAT fusion in C. gloeosporioides and C. siamense.  
a Percentage CAT fusion in different aged conidia viz. 6, 10, 13, 17 and 20 days of C. gloeosporioides and C. siamense 72 h post-incubation.  
b Percentage CAT fusion in 17 days old conidia of C. gloeosporioides and C. siamense incubated in water for different incubation time viz. 24, 48, 72 and 96 h.  
c Percentage CAT fusion in 17 days old conidia of C. gloeosporioides and C. siamense grown and incubated individually and in co-culture in water for 72 h.  
d Intra-specific CAT fusion in 17 days old C. gloeosporioides conidia.  
e Intra-specific CAT fusion in 17 days old C. siamense conidia.  
f Inter-specific CAT fusion between 17 days old C.
Figure 3

Different stages of inter-specific CAT fusion between *C. gloeosporioides* and *C. siamense* in-vitro. a CAT induction. b CAT homing. c CAT fusion. d CAT pre network. e and f CAT network. g detached conidia post CAT fusion. Arrow indicates CAT fusion, asterisk (*) symbol indicates *C. siamense* conidia and plus (+) symbol indicates conidia of *C. gloeosporioides*. Scale Bar = 20μm.
Figure 4

Effects of physiological stresses, nutrients, CAT inducers and CAT inhibitors on inter-specific CAT fusion frequency in C. gloeosporioides and C. siamense. Inter-specific CAT fusion percentage in water, PDB, oxidative stress (H2O2), osmotic stress (NaCl and sorbitol), heat, antifungal stress, glucose, KNO3, combination of glucose and KNO3, NaNO3, MgCl2, tryptophan, Insolution PD98059 (inhibitor of MAPKK pathway). Data from 3 replicates (n=300) and bar indicates standard deviation. Two asterisks (**) or three asterisks (***) indicate level of significance with a p value < 0.05 analyzed by ANOVA.
Figure 5

Inter-specific CAT fusion in *C. gloeosporioides* and *C. siamense* is mediated through quorum sensing phenomena. a Percentage CAT fusion in different conidial densities viz. $4 \times 10^2$, $4 \times 10^3$, $4 \times 10^4$, $4 \times 10^5$ and $4 \times 10^6$ of 17 days old conidia incubated in 1 ml of water (constant volume). B Percentage CAT fusion in young (6 days) and old (17 days) conidia in water, PDB, CAT medium supernatant and PDB+CAT medium supernatant. C Percentage CAT fusion in 17 days old conidia in CAT medium supernatant, chloroform...
treated CAT medium supernatant, proteinase-K treated CAT medium supernatant and water re-extract of chloroform treated CAT medium supernatant. Data from 3 replicates (n=300) and bar indicates standard deviation. Two asterisks (**) or three asterisks (***) indicate level of significance with a p value < 0.05 analyzed by ANOVA.

**Figure 6**

CAT mediated nuclear transfer between C. gloeosporioides and C. siamense. a, c, e and g Bright-field microscopy of CAT fusion between the 17 days old conidia C. gloeosporioides and C. siamense. b, d, f and h Visualization of nuclear transfer between the 17 days old conidia of C. gloeosporioides and C. siamense by DAPI staining using fluorescence microscope. Arrows indicate nucleus, asterisk (*) symbol indicates C. siamense conidia and plus (+) symbol indicates conidia of C. gloeosporioides. Scale bar = 20µm.
Figure 7

Movement of different cell organelles during CAT fusion between C. gloeosporioides and C. siamense. a Fluorescent, b Brightfield and c Overlapping microscopic images of mitochondrial movement. d Fluorescent, e Brightfield and f Overlapping microscopic images of lysosomal movement. g Fluorescent, h Brightfield and i Overlapping microscopic images of movement of lipid droplets. Arrow indicates CAT fusion, asterisk (*) symbol indicates C. siamense conidia and plus (+) symbol indicates conidia of C. gloeosporioides. Scale Bar = 10μm.
Figure 8

Colony morphology of C. gloeosporioides and C. siamense parent strains and their post-CAT fusion progenies. Colony morphology of parent C. gloeosporioides and C. siamense trains and their putative recombinants CG1-CG10 and CS1-CS10, respectively derived by single spore isolation from a mixture of conidia that had undergone CAT fusion.
Figure 9

AFLP analysis of C. gloeosporioides and C. siamense parent strains and their putative recombinant strains. a AFLP banding patterns of parent C. gloeosporioides (CGP), C. siamense (CSP) and putative recombinants of C. gloeosporioides (CG1-CG10). b AFLP banding patterns of parent C. siamense (CSP), C. gloeosporioides (CGP) and putative recombinants of C. siamense (CS1-CS10) along with 100bp DNA marker (M). Asterisk indicates the presence of extra bands beneath them.
Figure 10

Growth rate (cm) of C. gloeosporioides parent (CGP) and their recombinant strains (CG1-10) under different stresses. a Growth rate in presence of oxidative stress induced by H2O2. b Growth rate in presence of osmotic stress induced by NaCl. c Growth rate in osmotic stress induced by sorbitol. Two asterisks (**) or three asterisks (***), indicate level of significance with a p value < 0.05 analyzed by ANOVA.
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