**UNIVERSIDADE FEDERAL DE UBERLÂNDIA**

**INSTITUTO DE BIOTECNOLOGIA**

**PÓS-GRADUÇÃO EM BIOTECNOLOGIA**

**GUILHERME PEREIRA DE OLIVEIRA**

# *Trichoderma* spp. COMO POTENCIAIS AGENTES DE CONTROLE BIOLÓGICO PARA PODRIDÃO VERMELHA OCASIONADA POR *Colletotrichum falcatum* EM RESÍDUOS DE PÓS-COLHEITA DE CANA-DE-AÇÚCAR

**PATOS DE MINAS – MG**

**MARÇO DE 2020**

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Dissertação de Mestrado apresentada ao Programa de Pós-graduação em Biotecnologia como requisito parcial para a obtenção do título de Mestre em Biotecnologia.

**Professora Dra. Fernanda Marcondes de Rezende**

**PATOS DE MINAS – MG**

**MARÇO DE 2020**

Ficha Catalográfica Online do Sistema de Bibliotecas da UFU
com dados informados pelo(a) próprio(a) autor(a).

O48
2020

Oliveira, Guilherme Pereira de, 1993-
*Trichoderma* spp. como potenciais agentes de controle biológico para podridão vermelha ocasionada por *Colletotrichum falcatum* em resíduos de pós-colheita de cana-de-açúcar [recurso eletrônico] / Guilherme Pereira de Oliveira. - 2020.

Orientadora: Fernanda Marcondes de Rezende.
Dissertação (Mestrado) - Universidade Federal de Uberlândia, Pós-graduação em Biotecnologia.
Modo de acesso: Internet.
Disponível em: http://doi.org/10.14393/ufu.di.2020.116
Inclui bibliografia.
Inclui ilustrações.

1. Biotecnologia. I. Rezende, Fernanda Marcondes de,1981-, (Orient.). II. Universidade Federal de Uberlândia. Pós-graduação em Biotecnologia. III. Título.

CDU: 60

Bibliotecários responsáveis pela estrutura de acordo com o AACR2:
Gizele Cristine Nunes do Couto - CRB6/2091
Nelson Marcos Ferreira - CRB6/3074

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# ATA DE DEFESA - PÓS-GRADUAÇÃO

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|---|---|---|---|---|---|
| Programa de Pós-Graduação em: | Biotecnologia | | | | |
| Defesa de: | Mestrado Acadêmico | | | | |
| Data: | 16 de março de 2020 | Hora de início: | 13:30 h | Hora de encerramento: | 15:45 h |
| Matrícula do Discente: | 41812BTC003 | | | | |
| Nome do Discente: | Guilherme Pereira de Oliveira | | | | |
| Título do Trabalho: | *Trichoderma* spp. como potenciais agentes de controle biológico para podridão vermelha ocasionada por *Colletotrichum falcatum* em resíduos de pós-colheita de cana-de-açúcar | | | | |
| Área de concentração: | Biociências | | | | |
| Linha de pesquisa: | Bioinformática e biologia celular aplicada à genômica, transcriptômica e proteômica | | | | |
| Projeto de Pesquisa de vinculação: | | | | | |

Reuniu-se no Sala 203, Palácio dos Cristais, Campus Patos de Minas, da Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Biotecnologia, assim composta: Professores Doutores: Jorge Teodoro de Souza - UFLA/UCDavis; Terezinha Aparecida Teixeira - INGEB/UFU e Fernanda Marcondes de Rezende - FAMEV/UFU, orientadora da candidato.

Iniciando os trabalhos a presidente da mesa, Dra. Fernanda Marcondes de Rezende, apresentou a Comissão Examinadora e o candidato, agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

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Documento assinado eletronicamente por **Jorge Teodoro de Souza**, **Usuário Externo**, em 16/03/2020, às 15:58, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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**Referência:** Processo nº 23117.010886/2020-40 SEI nº 1875054

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Aprovado em ___/___/___

BANCA EXAMINADORA

_____

Prof. Dra. Fernanda Marcondes de Rezende

_____

Prof. Dr. Jorge Teodoro de Souza

_____

Prof. Dra. Terezinha Aparecida Teixeira

Patos de Minas – MG
2020

## DEDICATÓRIA

À Deus, família e professores, ofereço o resultado de meses de trabalho, no qual proponho ao mundo novas possibilidades.

## AGRADECIMENTOS

À Deus e a espiritualidade amiga que me permitiu seguir esse caminho evolutivo, e por propiciar tantas oportunidades de estudos colocando em meu caminho pessoas amigas e preciosas.

À minha mãe Liliane e ao meu pai Moacir pelas manifestações de apoio, carinho e palavras de encorajamento e amor.

Meu irmão Lucas, agradeço por ter contribuido efetivamente nos diversos processos dos experimentos que levaram a construção dessa dissertação.

As equipes do Marketing Técnico e de Pesquisa e Desenvolvimento da Lallemand por sugestionarem possibilidades relevantes ao trabalho.

Aos professores pelos ensinamentos, paciência, compreensão e competência.

Minha sincera gratidão aos amigos, ao meu namorado e às pessoas que sempre mantiveram incansáveis manifestações de incentivo.

Agradeço à minha orientadora Fernanda e ao meu caro colega de trabalho Alan pelas suas contribuições a este trabalho.

Não seria possível deixar de reconhecer a contribuição da usina WD e das equipes dos laboratórios da UFU Patos de Minas, em especial do Gbio (laboratório de Genética e Biotecnologia) e do LAGEM (laboratório de Genética Molecular).

## RESUMO

Nos últimos anos, a podridão vermelha ocasionada por *Colletotrichum falcatum* tem causado muitos danos à cultura da cana-de-açúcar, afetando a produtividade das lavouras canavieiras. O principal problema é que o patógeno permanece nos resíduos culturais e se multiplica, sendo uma importante fonte de inóculo para a próxima safra. Alternativas que podem reduzir a quantidade de inóculo de *C. falcatum* nos resíduos de pós-colheita são uma ótima ferramenta para serem integradas ao manejo da doença na cultura da cana-de-açúcar. Assim, o objetivo deste estudo foi avaliar o potencial de produtos a base de *Trichoderma* spp. como agentes de controle biológico da população de *C. falcatum* na palhada da cana-de-açúcar. O experimento foi conduzido em área de produção comercial de cana-de-açúcar localizada município de João Pinheiro, MG, Brazil. O delineamento experimental em blocos casualizados, com três tratamentos (controle e produtos a base de *Trichoderma asperellum* e *Trichoderma koningiopsis*) e sete repetições, foi implementado após a primeira colheita em área de cultivo da variedade RB867515. Duas coletas de amostras de resíduo pós-colheita foram realizadas, uma coleta inicial antes da aplicação dos produtos e, uma segunda coleta 30 dias após a aplicação dos tratamentos. A técnica de PCR em tempo real foi usada para quantificar o número de cópias de DNA de *C. falcatum*/µL nos resíduos pós-colheita. Os dados foram analisados pela análise de *deviance* em um modelo linear generalizado ajustado à distribuição Poisson e, as médias foram comparadas pelo teste de Tukey utilizando o pacote *multicomp* no software R. Os resultados da quantificação molecular demonstraram redução significativa do inóculo do patógeno ($p$-valor < 0,10) após a aplicação dos tratamentos a base de *T. asperellum* e *T. koningiopsis*. Uma vez que não houve diferença estatística na quantificação inicial do patógeno, a fórmula de Abbott foi aplicada e os tratamentos com *T. asperellum* e *T. koningiopsis* mostraram eficiência de 9,73% e 47,79%, respectivamente, na redução do crescimento de *C. falcatum* 30 dias após a aplicação sobre os resíduos pós-colheita da cana-de-açúcar. Portanto, demonstramos a eficácia de uma única aplicação de produtos a base de *Trichoderma* spp. como agentes de biocontrole da população de *C. falcatum* na cultura da cana-de açúcar. Além disso, nosso estudo mostrou que a técnica de PCR em tempo real é uma poderosa ferramenta de biologia molecular que pode ser usada para a avaliação da eficácia de agentes de controle biológico.



**Palavras-chave:** patógeno, PCR em tempo real, *Trichoderma asperellum* e *Trichoderma koningiopsis*

**ABSTRACT**

In the last years, red rot disease caused by *Colletotrichum falcatum* has caused a lot of damage to the sugarcane crop, affecting the yield. The main problem is that the pathogen remains in the crop residues and multiplies, being an important source of inoculum for the next crop year. Alternatives that may reduce *C. falcatum* inoculum in post-harvest residues are a great tool to be integrated into the management of the disease in sugarcane. Thus, the aim of this study was to evaluate the potential of products based on *Trichoderma* spp. as biological control agents against *C. falcatum* in post-harvest sugarcane residues. The experiment was conducted in a commercial sugarcane production area located in João Pinheiro, MG, Brazil. The experimental design in randomized blocks with three treatments (control and products based on *Trichoderma asperellum* and *Trichoderma koningiopsis*) and seven replicates was implemented after the first harvest in the cultivation area of the variety RB867515. Two collections of post-harvest residue samples were performed, an initial collection before application of products and, a second collection 30 days after the application of the treatments. qPCR technique was used to quantify the number of DNA copies of *C. falcatum*/µL in post-harvest residues. Analysis of deviance was performed under generalized linear model adjusted to Poisson distribution, and means were compared by Tukey test. The results of molecular quantification showed a significant reduction in the pathogen inoculum ($p$-value < 0.10) after application of treatments based on *T. asperellum* and *T. koningiopsis*. Since there was no statistical difference in the initial quantification of the pathogen, Abbott's formula was applied and the treatments with *T. asperellum* and *T. koningiopsis* showed efficacy of 9.73% and 47.79%, respectively, in reducing the growth of *C. falcatum* 30 days after application on post-harvest sugarcane residues. In summary, we demonstrate the efficacy of a single application of products based on *T. asperellum* and *T. koningiopsis* and their potential as a biocontrol agent for the *C. falcatum* population in post-harvest sugarcane residues. In addition, our study showed that the qPCR technique is a powerful molecular biology tool that can be used to evaluate the efficacy of biological control agents.

**Keywords:** pathogen, qPCR, *Trichoderma asperellum, Trichoderma koningiopsis*

**SUMÁRIO**

# 1 INTRODUÇÃO

O controle biológico tem crescido nos últimos anos e o uso de fungos e bactérias pode ser uma alternativa para o controle de pragas e doenças em diversas culturas. Dentre os microrganismos de maior destaque no controle biológico está o gênero *Trichoderma*. Este gênero é compreendido por fungos saprófitos de vida livre, que apresentam reprodução assexuada e podem ser encontrados em diferentes tipos de substratos. Esses microrganismos são essenciais para a manutenção das funções do solo devido sua participação em processos-chave como a formação da estrutura do solo, decomposição da matéria orgânica e ciclagem de nutrientes.

Além disso, os fungos do gênero *Trichoderma* também podem trazer inúmeros benefícios à planta. Dentre os quais se destacam a indução de resistência a patógenos, promoção de crescimento, mineralização de matéria orgânica no solo e inibição de crescimento de outros fungos por meio de diversos modos de ação, como por exemplo, parasitismo, competição e antibiose. Devido sua capacidade de rápida colonização da rizosfera, estabelecimento de populações estáveis e natureza competitiva, os fungos do gênero *Trichoderma* surgem como mais um componente ao manejo de doenças que ainda não possuem formas de controle eficiente, como é o caso da podridão vermelha.

A podridão vermelha é uma doença da cultura da cana-de-açúcar que ganhou destaque nos últimos tempos e causa grande impacto no teor de sacarose. Essa doença é causada pelo fungo *Colletotrichum falcatum*, cujos conídios infectam a planta por meio de lesões pré-existentes em diversos órgãos do vegetal. *C. falcatum* sobrevive em restos culturais por um longo período de tempo, desse modo a palhada proveniente da safra anterior é a principal fonte de inóculo do patógeno. Os conídios presentes nos restos culturais podem facilmente serem dispersos pelo ar, chuva, irrigação e insetos, contribuindo para a disseminação da doença.

O controle biológico surgiu como abordagem ecologicamente correta para superar os problemas causados pelos métodos químicos convencionais, pois além de apresentar especificidade ao alvo utiliza diversos modos de ação para atingi-lo. Ademais, por meio do controle biológico é possível interferir no ciclo de doenças contribuindo para a redução de sintomas e aumento de produtividade. Desse modo, o objetivo deste estudo foi avaliar o potencial de produtos a base de *Trichoderma* spp. como agentes de controle biológico da podridão vermelha (*C. falcatum*) na palhada da cana-de-açúcar por meio da técnica de PCR em tempo real.

# 2 REFERENCIAL TEÓRICO

## 2.1 *Trichoderma spp.*

O uso de microrganismos no controle de doenças, como a podridão vermelha, pode ser uma abordagem ecologicamente correta para superar os problemas causados pelos métodos químicos convencionais (VITERBO et al., 2002). Por meio do controle biológico é possível utilizar microrganismos específicos que interferem no ciclo de pragas e doenças contribuindo para a redução de sintomas e aumento de produtividade. Espécies do gênero *Trichoderma* provavelmente são as mais utilizadas no controle de doenças devido à sua natureza cosmopolita, pois estes possuem facilidade em colonizar diferentes tipos de substratos (DRUZHININA et al., 2011; ATANASOVA et al., 2013).

O gênero *Trichoderma* consiste de fungos filamentosos que pertencem à classe dos ascomicetos, dentre os quais se destacam *T. polysporum, T. viride, T. virens, T. asperellum, T. atroviride, T. koningii, T. lignorum, T. parceanamosum, T. stromaticum, T. pseudokoningii, T. koningiopsis* e *T. harzianum* (SAMUELS, 2006; DRUZHININA et al., 2011). Esses microrganismos são essenciais para a manutenção das funções do solo devido ao seu envolvimento em processos-chave como a formação da estrutura do solo, decomposição da matéria orgânica e ciclagem de nutrientes. Além disso, os fungos do gênero *Trichoderma* são decompositores de madeira e material herbáceo e, constituem o maior componente da microflora de vários ecossistemas (SEIDL, 2006).

Esses fungos são cultiváveis e apresentam rápido crescimento tanto *in vitro* quanto no campo. As colônias são inicialmente brancas e, após o desenvolvimento são observados tufos compactos verde-amarelados a verde-escuros muitas vezes apenas em pequenas áreas ou em zonas semelhantes a anéis concêntricos na superfície do meio de cultura. Seus conidióforos são ramificados, irregularmente verticilados, na sua maioria verdes e apresentam paredes lisas ou ásperas (ELLIS, 2018). Além disso, os fungos do gênero *Trichoderma* podem apresentar pleomorfismo, isto é, alguns deles possuem estágios morfológicos e fisiológicos diferentes. Sendo estes, estágio sexual (teleomórfico) e o estágio assexual (anamórfico ou mitospórico) (DRUZHININA et al,. 2010; SAMUELS et al., 2010).

Os fungos do gênero *Trichoderma* podem apresentar diversos modos de ação no combate a doenças causadas por microrganismos, como parasitismo, competição, antibiose e indução de resistência. No parasitismo, os fungos *Trichoderma* spp. atuam diretamente contra outros fungos digerindo e inativando suas estruturas por ações enzimáticas em hifas e estruturas

de resistência (GOMES et al., 2015). Na competição, os *Trichoderma* spp. disputam por espaço e nutrientes com os outros microrganismos (CASTRO et al., 2014). Por outro lado, na antibiose ocorre a liberação de compostos que irão inibir o crescimento (SILVA et al., 2019). Por fim, mas não menos importante, os fungos *Trichoderma* spp. promovem a indução de resistência em plantas estimulando a produção e secreção de moléculas elicitoras (ZEILINGER et al., 2016).

O uso de *Trichoderma* spp. pode trazer inúmeros benefícios a várias culturas, como por exemplo, indução de resistência no tomateiro à *Rhizoctonia solani* (MANGANIELLO et al., 2018) e a *Ralstonia solanacearum* (YENDYO et al., 2017), o controle de *Sclerotinia sclerotiorum* na cultura da soja (ZHANG et al., 2016) e de *Rhizoctonia solani* e *Pythium ultimum* na cultura do feijão (BRUNNER et al., 2005). Além de controlar doenças causadas por fungos, eles também possuem elevada capacidade metabólica, natureza competitiva, colonizam a rizosfera rapidamente, são capazes de estabelecer populações estáveis, promover o crescimento radicular e agir como bioestimulantes (GOMES et al., 2015). Ademais, fungos saprófitos, como os do gênero *Trichoderma,* podem contribuir com a decomposição de material orgânico favorecendo assim o processo de mineralização de nutrientes no solo (MAZA et al., 2014).

## 2.2 Cana-de-açúcar

A cana-de-açúcar pertence à família Poaceae e ao gênero *Saccharum*. O gênero foi descrito primeiramente em 1753 por Linnaeus no livro “Species Plantarum”, sendo também conhecido como *Sacchorifera* (CHEAVEGATTI-GIANOTTO et al., 2011). Esse gênero foi descrito com somente cinco espécies *S. officinarum, S. spontaneum, S. sinense, S. edule* e *S. barberi*, entretanto atualmente várias espécies são conhecidas (DILLON et al., 2007).

As cultivares mais utilizadas são híbridos derivados de cruzamentos, principalmente entre as espécies *S. officinarum* e *S. spontaneum* (DILLON et al., 2007). A espécie selvagem, *S. spontaneum,* é caracterizada pelo baixo teor de açúcar, talos finos, alto teor de fibra e elevada resistência a estresses bióticos e abióticos. Por outro lado, a espécie *S. officinarum*, denominada produtiva, possui alto teor de açúcar, talos grossos, baixo teor de fibra e baixa resistência a doenças (SINGH et al., 2010). Sendo assim, o resultado desse cruzamento é um híbrido produtivo com elevada resistência a patógenos.

A cultura da cana-de-açúcar é perene e as plantas apresentam caule do tipo colmo com vários metros de comprimento e suculento, apresentando altas concentrações de sacarose. O

sistema radicular da cana-de-açúcar é constituído por raízes adventícias e permanentes. As raízes adventícias emergem da zona da raiz do colmo e são responsáveis pela absorção de água durante a brotação e o suporte das plantas até que as raízes permanentes se desenvolvam (YANG et al., 2018). As raízes permanentes são fasciculadas e formam uma rede de raízes de absorção em vários eixos, normalmente ramificados e iguais na espessura e no comprimento, dificultando a distinção entre os eixos principal e secundário (CHEAVEGATTI-GIANOTTO et al., 2011).

A cana-de-açúcar é plantada em mais de 110 países e sua capacidade produtiva varia de área para área, sendo uma importante cultura de regiões tropicais e subtropicais (ÚNICA, 2012). No mundo, a área plantada de cana é de aproximadamente 24 milhões de hectares (SOUZA; SILVA, 2017), sendo o plantio destinado principalmente para produção de açúcar, etanol e bebidas.

A produção de cana-de-açúcar no Brasil na safra 2018/2019 foi de 622 milhões de toneladas em uma área plantada de 10 milhões de hectares, garantindo ao país posição de destaque como maior produtor. A maior região produtora de cana no Brasil é a Sudeste com quase de 436 milhões toneladas, representando 64 % da produção nacional (CONAB, 2019). A estimativa de produção para safra 2019/2020 é de 635 milhões de toneladas. O setor sucroalcooleiro também possui números positivos para exportação em 2020, estão previstos 17,2 milhões de toneladas de açúcar e 1,5 bilhões de litros de etanol (CNA, 2020), representando aumento de 35,4 e 35,1%, respectivamente, em relação as exportações de 2019 (CNA, 2019).

Dada a necessidade de mitigar os impactos ambientais e sociais causados pela cultura da cana-de-açúcar, o decreto 47.700 de 11/3/2003, que regulamenta a Lei no 11.241 de 19/9/2002 (SÃO PAULO, 2003), proibiu a realização de queimadas em canaviais no estado de São Paulo, seguindo um esquema de restrições legais progressivas até o ano de 2021 em áreas com possibilidade de mecanização total da colheita, e até 2031 para as demais áreas. Entretanto, a adoção de novas práticas agrícolas interfere, ao longo do tempo, na fitossanidade e, consequentemente, no manejo da cultura (DINARDO-MIRANDA; FRACASSO, 2013; GUIMARAES et al., 2008). De fato, tem-se observado um aumento na incidência de doenças fúngicas foliares na cultura da cana-de-açúcar, como a podridão vermelha.

### 2.3 *Colletotrichum falcatum*

*Colletotrichum falcatum* é o fungo causador da podridão vermelha, sendo essa uma das

principais doenças na cana-de-açúcar com incidência em vários países e que vem causando grandes perdas na produção sucroalcooleira (NAYYAR et al., 2017). A redução da produtividade está ligada ao fato do patógeno produzir a enzima invertase que causa a inversão da sacarose, isso interfere negativamente na recuperação de sacarose pela indústria (SHARMA; TAMTA, 2015). Estima-se que a podridão vermelha seja responsável pela perda de até 35 % do açúcar recuperável (VISWANATHAN, 2012).

A podridão vermelha pode se manifestar em diversas partes da planta dependendo do estágio de desenvolvimento. Durante a germinação, a doença pode provocar o apodrecimento de toletes e tecidos internos fazendo com que os mesmos apresentem coloração avermelhada, marrom ou cinza. Além de comprometer a germinação e o vigor, também pode provocar a morte das gemas. Nos colmos, a doença causa lesões internas de cor vermelha, que dão nome à doença (RAGO; TOKESHI, 2005). Por outro lado, nas folhas os sintomas podem surgir na forma de lesões nas nervuras centrais, sendo que o tamanho das lesões é variável, podendo atingir toda a extensão da nervura (DEL PONTE, 2007).

*C. falcatum* apresenta hifas finas, incolores, ramificadas e septadas que podem infectar o hospedeiro de forma inter e intracelular. As hifas também produzem estruturas de resistência chamadas clamidósporos, que são propágulos vegetativos altamente resistentes capazes de sobreviver em condições adversas por um longo período de tempo (SUNGANDHA, 2018). Os esporos são as principais fontes de dispersão do fungo, pois podem ser facilmente dispersos por meio de correntes de ar, chuva, sistema de irrigação e insetos (MEJHAK, 2018).

A doença se desenvolve a partir de fontes de inóculo primário e secundário. As fontes de inóculo primário são antigos caules e folhas fragmentados e outros detritos sobre os quais o fungo cresce de forma saprófita (MEJHAK, 2018). Essa é a principal forma de transmissão do patógeno, sendo os restos culturais um problema sério para a contaminação da cultura da cana-de-açúcar. Por outro lado, as fontes secundárias de inóculo são as nervuras centrais de folhas doentes, onde a contaminação ocorre pelos conídios que são disseminados no ambiente por meio de agentes bióticos e abióticos (SUNGANDHA, 2018). Inúmeros fatores podem contribuir para a incidência da doença, como por exemplo, crescimento tardio da planta hospedeira por falta de operações culturais adequadas, cultivo contínuo da mesma variedade de cana-de-açúcar em uma determinada área ou até mesmo cultivo de variedades suscetíveis em áreas vizinhas (MEJHAK, 2018).

A prática de controle mais utilizada para a podridão vermelha é o emprego de variedades resistentes, pois o emprego de produtos químicos não é eficiente (ASHWIN et al., 2017). Outra forma de manejo da doença é por meio do controle da broca-da-cana, pois esse inseto causa

lesões à planta e essas são a principal via de entrada do fungo no colmo da planta (SANTOS, 2010). Na literatura, o uso de fungos do gênero *Trichoderma* se mostrou eficiente no controle de *C. falcatum* em testes de cultivo pareado pela produção de compostos não voláteis, pela aplicação de metabólitos secundários sob condições de campo e pela redução da severidade em casa de vegetação e no campo (JOSHI; MISRA, 2013; Joshi et al., 2016; SINGH et al., 2008). Sendo assim, o uso de agentes biológicos como fungos do gênero *Trichoderma* pode ser uma alternativa para a redução da população do *Colletotrichum falcatum* na cultura da cana-de-açúcar. Entretanto, ainda não existem estudos utilizando ferramentas de biologia molecular que avaliaram a eficácia de fungos do gênero *Trichoderma* no controle do agente causador da podridão vermelha.

# *Trichoderma* spp. as potential biological control agents for *Colletotrichum falcatum* population in post-harvest sugarcane residues

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## ABSTRACT

In the last years, red rot disease caused by *Colletotrichum falcatum* has caused a lot of damage to the sugarcane crop, impacting the yield. Alternatives that can reduce *C. falcatum* inoculum in crop residues are great tools to be integrated into the management of the disease in sugarcane. Thus, the aim of this study was to evaluate the potential of products based on *Trichoderma* spp. as biological control agents against *C. falcatum* in post-harvest sugarcane residues. The trial was conducted in a commercial sugarcane production area in a completely randomized block design with three treatments and seven replicates. Analysis of deviance was performed under generalized linear model adjusted to Poisson distribution, and means were compared by Tukey test. Quantification showed a significant reduction in the pathogen inoculum after application of treatments based on *Trichoderma asperellum* and *Trichoderma koningiopsis*. Abbott's formula was applied and the treatments with *T. asperellum* and *T. koningiopsis* showed efficacy of 9.73% and 47.79%, respectively, in reducing the growth of *C. falcatum* at 30 days after application on post-harvest sugarcane residues. Overall, our findings indicated the efficacy of products based on *Trichoderma spp*. against *C. falcatum*, and their potential as biocontrol agents in sugarcane crop. In addition, the qPCR proved to be a powerful tool of molecular biology in the evaluation of biological control trials, since it allows the quantification of specific microorganisms in the environment. These results have the potential to help the sugar and alcohol industry to reduce the yield losses caused by *C. falcatum*.


**Keywords:** pathogen; qPCR; Trichoderma asperellum; *Trichoderma koningiopsis*

## Declarations

*Funding*

This work was supported by Laboratório Farroupilha Lallemand.

*Conflicts of interest/Competing interests*

The authors have no competing interests to declare.

*Ethics approval*

Not applicable.

*Consent to participate*

Not applicable.

*Consent for publication*

Not applicable.

*Availability of data and material*

All relevant data are within the manuscript. Datasets and scripsts used in the analyses are available from the corresponding author under reasonable request.

*Code availability*

Not applicable.

*Authors' contributions*

AWVP conceived the idea; GPO, TGA, AWVP and FRM designed the study; GPO conducted the experiment, performed data analysis and drafted the manuscript. TGA, AGS, ICM, DCB and TAT collaborated in laboratory analyses. TGA, AWVP and FRM provided data analysis support and were involved in the interpretation of the results. All authors have read and approved the final manuscript.

# INTRODUCTION

Sugarcane is one of the most important agro-industrial crop in the world, mainly for its sugar source, being used for sugar, bioethanol and alcoholic drinks production (Patel et al. 2018). However, the crop is affected by many fungal diseases that cause considerable yield losses in sugar content. Probably, red rot is one of the major disease affecting sugarcane worldwide (Viswanathan et al. 2016).

*Colletotrichum falcatum* is the fungus that causes red rot, one of the main diseases in sugarcane with incidence in several countries, causing great losses in sugar and alcohol production (Nayyar et al. 2017). The decreasing in yield is related to the fact the pathogen produces the invertase enzyme that causes sucrose inversion, which negatively interferes with sucrose recovering by the sugar and alcohol industry (Sharma and Tamta 2015). Red rot disease can manifest in different parts of the plant depending on the stage of development of the plant. It is noteworthy that the main source of pathogen inoculum is the post-harvest residues, which is a serious problem for the contamination of the sugarcane crop (Mejhak 2018).

Resistant varieties is the most applied control practice for red rot, as the use of chemical products is not efficient (Ashwin et al. 2017). However, there is still a need to implement other ways of managing the disease development. In this sense, biological control can be an environmentally friendly approach to the red rot management. Biological control allows using specific microorganisms that interfere in the diseases cycle, contributing to reduce the symptoms and increase yield. Species of the genus *Trichoderma* are probably the most used in disease control due to their cosmopolitan nature, because they easily colonize different types of substrates (Atanasova et al. 2013; Druzhinina et al. 2011).

The genus *Trichoderma* can show several modes of action against diseases caused by fungi, such as parasitism (Gomes et al. 2015), competition (Castro et al. 2014), antibiosis (Silva et al. 2019) and induction of resistance (Zeilinger et al. 2016). In addition, they also have high metabolic capacity, present competitive nature, colonize the rhizosphere quickly, are able to establish stable populations, promote root growth and act as biostimulants (Gomes et al. 2015). Indeed, previous studies described the benefts of *Trichoderma* spp. on resistence induction of tomato plant to *Rhizoctonia solani* (Manganiello et al. 2018) and *Ralstonia solanacearum* (Yendyo et al. 2017) and, on the population control of *Sclerotinia sclerotiorum* in soy (Zhang et al. 2016), *Rhizoctonia solani* and *Pythium ultimum* in bean (Brunner et al. 2005), and *Colletotrichum gloeosporioides* in papaya (Ribeiro et al. 2016). Furthermore, saprophytic fungi such as those of the genus *Trichoderma* can contribute to the decomposition of organic material, favoring the process of nutrient mineralization in the soil (Maza et al. 2014).

To the best of our knowledge, no study to date has explored the use of molecular biology tools to evaluate the feasibility of *Trichoderma* spp. as biological control agent against *C. falcatum* in post-harvest residues of sugarcane. Therefore, the aim of this study was to evaluate the potential of products based on *Trichoderma* spp. as biological control agents against *C. falcatum* in the sugarcane crop residue using real-time PCR.

## MATERIAL AND METHODS

### Site description and experimental design

The experimental area is situated on a commercial sugarcane crop in João Pinheiro, located in the northwest portion of Minas Gerais, Brazil, at 18°07'19.6"S, 45°57'02.9"W. The total cultivated area covers about 28 thousand hectares; however, the field trial was set up in 13.78

hectares of RB867515 variety under dryland cropping system. The experimental design comprising seven complete randomized blocks with three treatments (control and products based on *Trichoderma asperellum* and *Trichoderma koningiopsis*) and seven replicates was established on May 8th, 2019, after the first harvest, i.e., 14 months after planting.

The commercial formulation of two products based on *Trichoderma* spp. was sprayed once on the day the trial was set. The microorganisms belonging to the official Lallemand Plant Care Collection are registered under the codes *Trichoderma asperellum* LFCC0087 and *Trichoderma koningiopsis* GF 427. The dose applied is recommended by the manufacturer for the control of soil borne diseases (Table 1). The application was carried out using Husqvarna gasoline sprayer model 321S25 adapted with a 6-nozzle bar type AI-11002 with 50 cm spacing of each nozzle and a spray volume of 300 L/ha. The Break-Thru® adjuvant was used at a dose of 1 mL/L. The control plots followed exclusively the standard management of sugarcane cropping system (i.e., growth regulators, herbicides, insecticides and fertilizers), and they were sprayed with only water and adjuvant using the same spray volume to guarantee the identical amount of liquid received on the other plots.

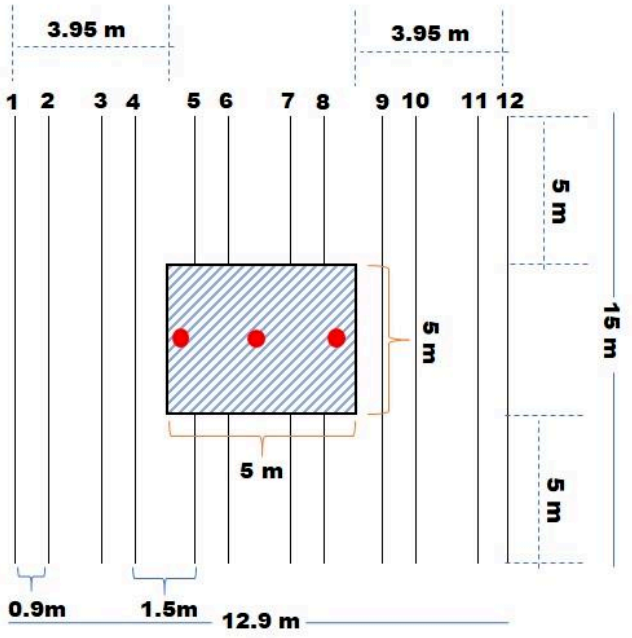
**Table 1** Treatments description

| Treatment | Description | Dose (g/ha) | Product concentration (cfu/g) |
|---|---|---|---|
| T1 | Control | - | - |
| T2 | *T. asperellum* LFCC0087 | 400 | $1x10^{10}$ |
| T3 | *T. koningiopsis* GF 427 | 400 | $3x10^{9}$ |

Each plot covered an area of 12.9 x 15 m containing 12 planting rows spacing 0.9 m within double rows, and 1.5 m between the double rows. A collecting sample area of 25 $m^2$ (5 x 5 m) was delimited in the middle of the plot to avoid a cross contamination during the spraying of

the products. In addition, the blocks were 0.8 m apart. Three samples were collected from each plot to ensure adequate sampling of the area (Fig.1). Samples were taken at the interface between soil and crop residue, given the ability of fungal species to colonize mainly the soil and the first centimeters of crop residues. In fact, many factors, such as temperature, humidity, organic matter and nutrients, make this environment favorable to the development and growth of fungi (Harman et al. 2004; Xian et al. 2020).

**Fig. 1** Plot scheme




Plots scheme showing 12 planting rows and the spacing between them. Collecting sample area of 25 $m^2$ (blue) with the red dots representing the samples collection spots

## DNA extraction from crop residues

Crop residues samples were collected on 2 different times. First sample was collected before the application of *Trichoderma* spp., and the second, 30 days after the application of the treatments. Samples from the same plot were homogenized within each period, and then three

154 sub-samples (3 g each) from the composite sample was weighted, individually identified and
155 stored at -80 °C. Prior DNA extraction, samples were lyophilized at -51 °C for 72 hours to
156 dehydrate and triturated with the aid of a mortar and pistil to increase the surface contact. Since
157 it was the first time that the protocol developed by Souza and Teixeira (2019) was used to
158 extract DNA from post-harvest residues samples, some adaptations, such as, the amout of
159 sample triturated, extraction buffer volume, adding a step of cleaning DNA after extraction and
160 the use of RNase, were done. The DNA quantification and qualification were performed using
161 Bel Photonics UV-M51 UV-VIS Spectrometer and agarose gel electrophoresis technique (0.8
162 %), and DNA samples were stored at −20 °C until use. Unless otherwise stated, total DNA
163 extracted from sugarcane residues samples was diluted in Milli-Q water to 25 ng of total DNA
164 per µL for qPCR analysis.
165
166 **qPCR conditions**
167 Real-time qPCR amplification and analysis were performed using a StepOnePlus™ Real-Time
168 PCR System with software version 2.2.2. Forward 5'-CCTACCCAACCGAGTATCG-3' and
169 reverse 5'-GCGCAGCTTGCTCTCAAGAGC-3' primers described as amplifying a SCAR
170 sequence of 442 pb from *C. falcatum* genome (Chandra et al. 2015; Nithya et al. 2012) were
171 used. *In silico* and *in vitro* checking of primers specificity were performed. All qPCR assays
172 were implemented in replicates in a 10 µL reaction volume containing 5µL PowerUp™ SYBR®
173 Green Master Mix (Thermo Fisher, CA, USA), 0.5 µL primers (final concentration 0.5µM), 2.5
174 µL of DNAse-free water and 50 ng of DNA. The thermal cycling protocol was as applied: an
175 initial denaturation for 10 min at 95 °C, followed by 40 amplification cycles of 15 s denaturing
176 at 95 °C and 1 min annealing-extension step at 60 °C. After the amplification, a melting curve
177 analysis with a temperature gradient of 0.5 °C/s from 60 to 95 °C was performed to confirm
178 lack of nonspecific PCR amplification and dimer formation. Finally, the samples were cooled

179 down to 25 °C for 0.5 °C/s. In addition, each run included no-template controls.

180

181 **Construction of standard curves and quantification**

182 Conventional PCR was carried out in a 20 µL reaction with real-time PCR primers (0.5 µM), 1
183 U of Platinum Taq® DNA Polymerase Hotstar (Thermo Fisher, CA, USA), 1 X PCR buffer
184 (0.1 mM EDTA, 1 mM DTT, 50% v/v glycerol, 20 mM Tris-HCl, pH 8.0 ), 1.5 Mm $MgCl_2$, ,
185 200 µM of dNTP mix solution and 100 ng of DNA template. Cycling conditions consisted of
186 an initial denaturation at 95 ºC for 4 min, then 28 cycles composed of the following steps:
187 denaturation at 94 °C for 40 seconds, annealing at 59 °C for 40 seconds and extension at 72 °C
188 for 50 seconds. Finally, a final extension of 72 ºC was performed for 10 min. Then, the reaction
189 product was evaluated by 1.5% agarose gel electrophoresis.

190

191 The PCR product was purified using QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany)
192 and cloned into pCR™4-TOPO® TA vector (Thermo Fisher, CA, USA) according to the
193 manufacturer's instructions. The recombinant vector was transformed into competent *E. coli*
194 and 50 µl of transformed culture was spread onto Petri dishes plates containing ampicillin. Only
195 transformed (white) colonies grown on the media, thus they were picked and processed for
196 plasmid isolation. Plasmid purification was done using an UltraClean® 6 Minute Mini Plasmid
197 Prep Kit (Qiagen, Hilden, Germany). Plasmid linearization was done by restriction digestion
198 using Fast Digest PstI (Thermo Fisher, CA, USA). Digestion products were detected by
199 electrophoresis agarose (1%), the linearized plasmid was quantified using a Bel Photonics UV-
200 M51 UV-VIS Spectrometer and the number of copies/ µL was calculated for all standards as
201 $\frac{6.022*10^{23}*\text{DNA concentration}}{\text{number of bases pairs}*660}$ (Godornes et al. 2007).

202

A 10-fold serial dilution series ranging from 1 x $10^2$ to 1 x $10^7$ copies of DNA/µL was used to construct the standard curves. The quantification allows to determine the actual copy numbers of target sequences by relating the $C_q$ value to a standard curve (Lilly and Barnett 1951). In this sense, qPCR efficiency plays an important role in samples quantification. $C_q$ values in each dilution were measured in quadruplicate and were plotted against the logarithm of their initial template copy numbers. Each standard curve was generated by a correlation coefficient ($R^2$) of the plotted points. From the slope of each standard curve, PCR amplification efficiency (E) was calculated using $E = 10^{(-1/slope)} - 1$ (Lee et al. 2006).

**Statistical analysis**

The number of *C. falcatum* DNA copies/µL was given by StepOnePlus™ Real-Time PCR System version 2.2.2 based on the standard curves constructed. The data for both collections, before and 30 days after application of products based on *Trichoderma* spp. in post-harvest sugarcane residues, were submitted to the normality and homogeneity tests. Given these principles were not reached, analysis of deviance was performed. In this case, the generalized linear model (GLM) was adjusted to Poisson distribution, and the means were compared by Tukey test. The analysis of deviance is a close analog to the analysis of variance (ANOVA), which uses residual deviance to evaluate the significance of the model. The residual deviance is also analogous to the ANOVA residual sum of squares. The residual deviance for GLM is $D_m = 2(log_e L_s - log_e L_m)$, where $L_m$ is the maximized likehood under the model in question and $L_s$ is the maximized likelihood under a satured model (Fox 2016). GLM was adjusted to Poisson distribution, due to the fact this distribution shows how many times an discrete event is likely to occur within a specified period of time (Kissell and Posserina 2017) (i.e., number of the *C. falcatum* DNA copies). Statistical analyses were performed using the R package *multicomp* and the level of significance was set at $\alpha = 0.10$. Abbott's formula was used

228 to evaluate the efficacy of *Trichoderma* spp. (Abbott 1925).

229

230 **RESULTS**

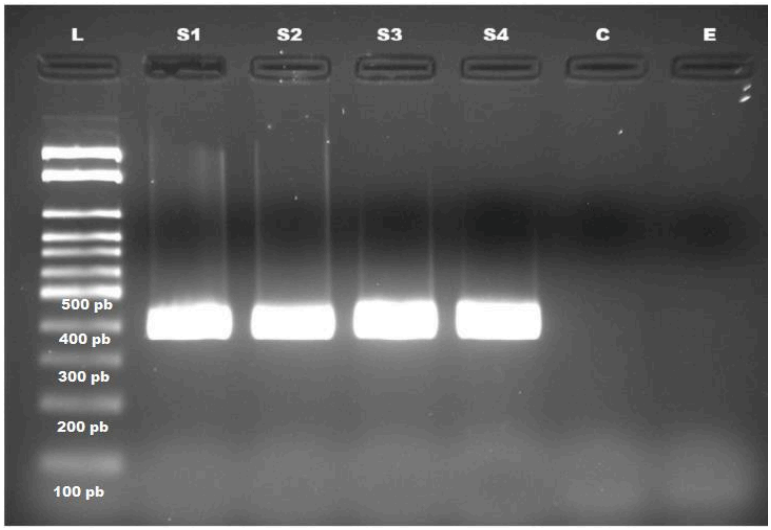
231

232 **Primers specificity checking**

233 Analysis *in silico* is the first step to check primers specificity, because it is a simple and free
234 analysis. Thus, the pair of primers were confronted to the NCBI genome databases. The results
235 showed a low e-value ($4 \times 10^{-6}$) and percentage of similarity and query cover of 100% to the *C.*
236 *falcatum* genome. The second step is to verify the specificity *in vitro*. For that, the DNA was
237 extracted from pure *C. falcatum* mycelium grown on Petri dishes. After the extraction,
238 conventional PCR was perfomed, and electrophoresis gel was used to confirm the fragment of
239 442 pb amplification. As expected, the bands on the gel were well defined and corresponding
240 to the 442 pb fragment (Fig. 2). This amplicon was also used to construct the standard curves.

241

242 **Fig. 2** Gel electrophoresis for 442 pb fragment from *C. falcatum* genome




243

244 Gel electrophoresis of the PCR products were performed on 1.5 % agarose
245 gel. L = DNA ladder of 100 pb; S1, S2, S3, S4 = 442 pb amplicon for *C.*
246 *falcatum* genome replicates, C and E = negative controls

247 **qPCR efficiency and standard curves**

248 The standard curve for *C. falcatum* primers ranging from 1 x $10^2$ to 1 x $10^7$ DNA copies/µL was
249 linear ($R^2$ > 0.992) by at least duplicate reactions, the values found for slope was -3.359, and
250 threshold 0.078284. The cycle quantification ($C_q$) value deviation was calculated for the 10-
251 fold serial dilutions from the assays performed. It is important to compare the $C_q$ value between
252 qPCR assays to assure the reaction occurred under identical conditions. By comparing the $C_q$
253 values of samples of unknown concentration with a series of standards is possible to determine
254 the amount of template DNA in an unknown reaction (ThermoFisher 2016). The $C_q$ values
255 obtained from the assays in the established ranging were stable, the average of $R^2$ and efficiency
256 (%) were also very high as presented on Table 2.

257

258 **Table 2** Validation results for *C. falcatum* qPCR assays

| Linear range of fungal DNA (ng/µL) | $C_q$ | $R^2$ | Efficiency (%) |
|---|---|---|---|
| $10^2$ | 29.62 ± 0.47 | 0.992 ± 0.005 | 99.778 ± 1.61 |
| $10^3$ | 26.35 ± 0.20 | | |
| $10^4$ | 23.28 ± 0.31 | | |
| $10^5$ | 20.13 ± 0.41 | | |
| $10^6$ | 16.75 ± 0.48 | | |
| $10^7$ | 12.55 ± 0.22 | | |

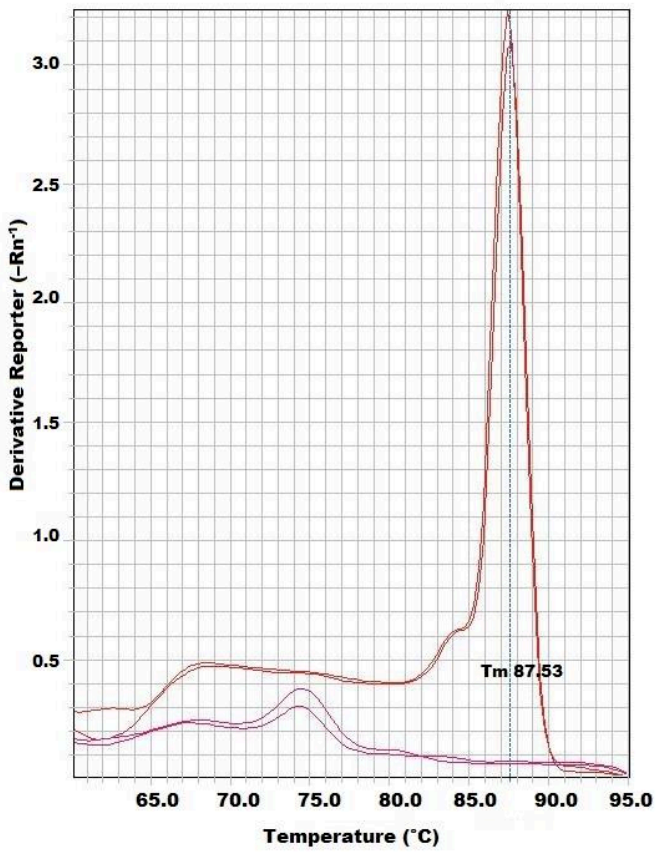
259 $C_q$, $R^2$ and Efficacy are reported as mean values ± standard deviation of at least two biological replicates

260

261 The pair of primers specificity was also confirmed in qPCR by melting curving analysis. The
262 PCR product of the DNA samples showed a sharp peak, at 87.53 °C. The results indicated that
263 none non-specific PCR products were amplified with the *C. falcatum* primers (Fig. 3).

264

**Fig. 3** Melting peak for PCR product

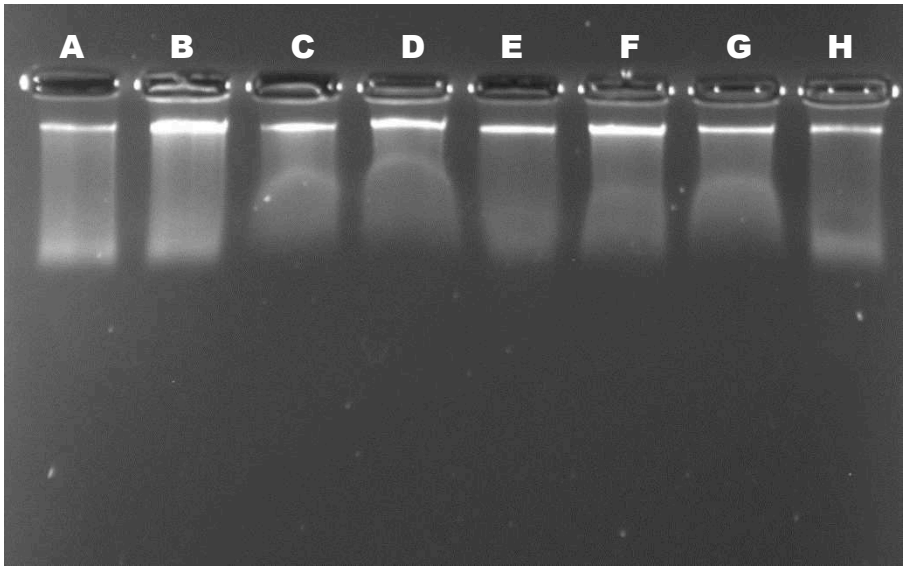



Melting peak were examined for all DNA samples. The melting temperature (Tm) was 87.53°C

## Monitoring of *C. falcatum* in post-harvest sugarcane residues

For monitoring of *C. falcatum* population in post-harvest sugarcane residues before and after application of products based on *Trichoderma* spp. the quality of the DNA extracted was checked by gel electrophoresis before quantification for all samples. Fig. 4 illustrates the mininum DNA extraction quality adopted for of all the 126 samples (3 treatments x 7 replicates x 3 sub-samples x 2 collections) to avoid interferences in qPCR technique. Following, qPCR analyses were performed to determine the efficacy of products based on *Trichoderma* spp. in reducing *C. falcatum* population under field conditions.

279 **Fig. 4** Examples of standard of quality for DNA extracted




280

281 Gel electrophoresis of the extracted DNA were performed on 0.8% agarose gel to

282 check the DNA quality. A, B, C, D, E, F, G, H = DNA samples

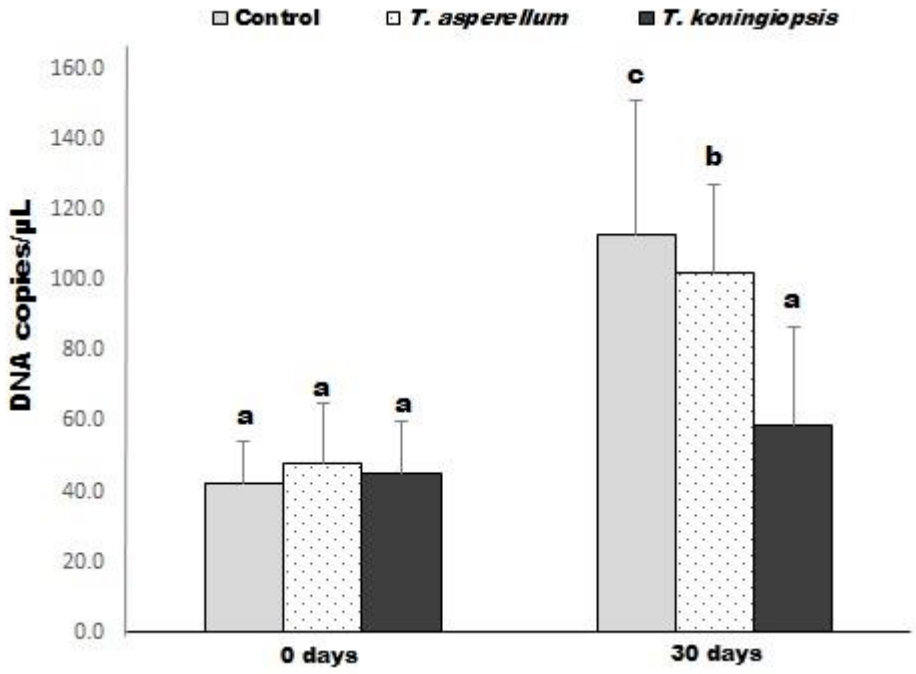
283

284 Fig. 5 shows the number of *C. falcatum* DNA copies/µL before and 30 days after the application
285 of products based on *Trichoderma* spp. on post-harvest sugarcane residues. Note that there was
286 no statistical difference between treatments before products application, demonstrating
287 uniformity of *C. falcatum* population. However, a significant reduction on the pathogen
288 population was observed 30 days after application of *T. asperellum* ($p$-value $< 0.10$) and *T.*
289 *koningiopsis* ($p$-value $< 0.01$) treatments compared with control. Abbott's formula showed
290 efficacy of 9.73% and 47.79% for *T. asperellum* and *T. koningiopsis* on reducing *C. falcatum*
291 population, respectively.

292

293 **Fig. 5** Number of DNA copies/µL of *C. falcatum* before and after the

294 application of products based on *Trichoderma* spp.




Data are reported as mean values and letters indicate significant difference between treatments by deviance analysis under generalized linear model adjusted to Poisson distribution, followed by Tukey test ($p$-value < 0.10). The means represented on the figure were calculated using 42 samples (7 replicates x 3 sub-samples x 2 duplicates) per treatment in each sampling period

## DISCUSSION

Applying a tool to identify and quantify pathogens in post-harvest residues can lead advances in the biological control area. Through specific primers is possible to determine the amount of pathogens present in crop residues and soils and understand their dynamics in the environment (Legrand et al. 2018). Many authors have been demonstrating efficacy in methodologies involving pathogen quantification by qPCR technique in different crops, such as, the cereal pathogen *Fusarium graminearum* (Legrand et al. 2018), *Colletotrichum camelliae* in tea plant (*Camellia sinensis*) (He et al. 2020) and *Xanthomonas fragariae* in strawberry (Wang and Turechek 2020). Thus, the qPCR technique can play an important role in quantifying pathogens

312 in post-harvest sugarcane residues, such as *C. falcatum*. Here, we used qPCR to evaluate the
313 number of DNA copies of *C. falcatum* in sugarcane residues. Previously, the antagonic effect
314 of *Trichoderma* spp. against C. *falcatum* was reported in the literature. Dual culture laboratory
315 assays showed the growth inhibiting effect of non-volatile compounds produced by
316 *Trichoderma* spp. (Joshi and Misra 2013). Furthermore, red rot incidence reduced after the
317 application of secondary metabolites produced by *Trichoderma* spp. under field conditions
318 (Joshi et al. 2016). In addition, *Trichoderma* spp. treatments reduced the disease severity in
319 glass house and field conditions (Singh et al. 2008). Although, to the best of our knowledge our
320 study was the first to use the qPCR tool as a molecular approach to evaluate the efficacy of
321 *Trichoderma* spp. against *C. falcatum* population under field conditions.
322
323 Initially, the pair of primers (Chandra et al. 2015; Nithya et al. 2012) were validated *in silico*
324 and *in vitro* and it showed specificity to amplify *C. falcatum* 442 pb fragment (Fig.1). The
325 genomic region amplified by the primers is a SCAR (sequence characterized amplified region)
326 marker, which is characterized by a defined and specific genomic DNA fragment that can be
327 detected by PCR using a pair of specific primers (Paran and Michelmore 1993). SCAR markers
328 are extremely reliable because they are highly specific, and have been widely used in the
329 identification of microorganisms (Priyanka et al. 2014; Marieschi et al. 2016).
330
331 After the primers' validation, we carefully constructed the standard curve to be able to perform
332 *C. falcatum* quantification. The qPCR parameters showed high efficiency, confirming that the
333 quantification for the samples relative to the standard curves were trustable (Table 2). For
334 reliable analysis, construction of standards and also storage conditions should be taken in
335 account to ensure more robustness and suitable quantification (Dhanasekaran et al. 2010).
336 Another step, that we carefully checked, was the DNA extraction quality (Fig. 4). DNA quality

337 is very important, because in case of high DNA degradation or high content of RNA, the
338 quantification may suffer interferences. Although, some degradation in the DNA samples was
339 observed, it did not interfere in the qPCR quantification. The DNA degradation is frequently
340 observed due to procedures involving DNA extraction (Chen et al. 2020), such as cell lysis and
341 removal of impurities, leading to chromosomal breaks through mechanical agitation and sample
342 handling (Miller et al. 1988).
343
344 After checking the DNA samples quality, qPCR analyses were performed to obtain the number
345 of *C. falcatum* DNA copies/µL for both sampling periods (Fig. 5). In the first, there was no
346 statistical difference between the treatments. However, *T. asperellum* and *T. koningiopsis*
347 products reduced *C. falcatum* population compared to control at 30 days after application, and
348 this reduction was greater in *T. koningiopsis* treatment. It is important to remark that *C. falcatum*
349 population increased from the first to the second sampling period in all treatments, indicating
350 that the pathogen was growing. It's known that the pathogen remains as dormant until finds
351 conditions to grow, and high humidity and lower temperature are great conditions for *C.*
352 *falcatum* to start the colonization (Patel et al. 2019). Thus, probably after application of the
353 products the humidity increased, and the temperature decreased contributing to the pathogen
354 multiplication in the crop residue. While the initial inoculum remained in the crop residue will
355 be the source of inoculum for the next crop year (Mejhak 2018), treatments based on
356 *Trichoderma* spp. appear to be promising alternatives for controlling the *C. falcatum* population
357 in post-harvest sugarcane residues.
358
359 Given that the initial number of *C. falcatum* DNA copies was uniform we applied the Abbott's
360 formula to calculate the efficacy of *Trichoderma* spp. Efficacy of 9.73% and 47.79% for *T.*
361 *asperellum* and *T. koningiopsis* on reducing *C. falcatum* population was observed, respectively.

362 *Trichoderma* strains can dominate a wide variety of habitats, due to their high metabolic
363 capacity and aggressive competitive nature (Kubicek et al. 2008; Lopes et al. 2012). All these
364 features help *Trichoderma* to be a powerful biocontrol agent against many pathogens. This
365 difference of efficacy between the treatments can be explained by the different characteristics
366 of each species of *Trichoderma,* some species, such as, *T. harzianum* and *T. koningiopsis* can
367 be highly adaptable to the environment and show higher aggressiveness (Chang et al. 2020).
368 The dose applied for *T. asperellum* and *T. koningiopsis* was the same (400 g/ha), however the
369 products concentration was different, *T. asperellum* concentration was 3.33 times higher than
370 *T. koningiopsis.* We followed the manufactors recommendations and did not adjust the dose
371 rate. This also confirm *T. koningiopsis* high efficacy, because its concentration was lower than
372 *T. asperelum* and still performed better.
373
374 *Trichoderma* spp. showed efficacy by preventing *C. falcatum* population increasing.
375 *Trichoderma* spp. can present several modes of action against pathogens and can be used as
376 biocontrol agents (Silva et al. 2019). Through antibiosis *Trichoderma* spp. produces secondary
377 metabolites with anti-fungal activity (Silva et al. 2019). In parasitism, the pathogen is affected
378 by cell wall-degrading enzymes (Gomes et al. 2015), a competition for nutrients or space can
379 also be an important mode of action (Castro et al. 2014). For the last, but not least, induction of
380 resistance in plants through the production and secretion of elicitor molecules (Zeilinger et al.
381 2016).
382
383 It is important to highlight that the methodology used to quantify *C. falcatum* in post-harvest
384 crop residues took into account the total pathogen DNA present on the samples, thus we did not
385 differ DNA recovered from living, dead or dormant structures (Lievens et al. 2006; Legrand et
386 al. 2018). To overcome this limitation, an alternative would be to treat the samples with

propidium monoazide (PMA) before the freezing process for sample storage (Hjortkjaer et al. 1986). PMA can avoid any DNA amplification from dead cells, because it is a photo reactive dye with high affinity for DNA double strain, thus the dye intercalate into DNA and form a covalent linkage upon exposure to intense visible light, inhibiting PCR amplification (Scariot et al. 2018; Soto-Munoz et al. 2014; Biotium 2020).

In summary, our study showed the efficacy of a single application of products based on *Trichoderma* spp. in reducing *C. falcatum* population in post-harvest sugarcane residues, and their potential as biocontrol agents. In addition, we proved that qPCR technique can be a powerful molecular tool to be used to evaluate the efficacy of biological control agents, since it allows quantification of specific microorganisms in the environment. These results have the potential to help the sugar and alcohol industry to reduce the yield losses caused by *C. falcatum*.

## ACKNOWLEDGEMENTS

We acknowledge financial support by the Laboratório Farroupilha Lallemand. The authors thank the WD Agroindustrial for providing the experimental area.

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## 3 CONCLUSÃO

Evidenciou-se a eficácia de uma única aplicação de produtos a base de *Trichoderma* spp. na redução da população de *C. falcatum* em resíduos de pós-colheita, bem como seu potencial como agentes de biocontrole. Além disso, provamos que a técnica de PCR em tempo

real pode ser uma poderosa ferramenta de biologia molecular a ser utilizada para avaliar a eficácia de agentes de controle biológico, uma vez que permite a quantificação de microrganismos específicos no ambiente. Até onde sabemos, fomos os primeiros a usar esta técnica para demonstrar a eficácia de *T. asperellum* e *T. koningiopsis* na redução da população de *C. facaltum* em resíduos de pós-colheita de cana-de-açúcar sob condições de campo. Esses resultados têm potencial de colaborar com a indústria sucroalcooleira na redução das perdas de produtividade causadas pelo *C. falcatum*.

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## ANEXO I - European Journal of Plant Pathology - Instructions for Authors Sequences

Sequences must be deposited in the GenBank, full alignment of datasets must be submitted to the TreeBASE and a Mycobank number must be added for new species and taxonomic changes.

Please submit Sequences as Electronic Supplementary Material at reviewing stage.

### Types of papers

All contributions should be submitted in English and are subject to peer reviewing.

Research papers describing original research should address biological problems. They should contain a novel and well formulated hypothesis, a sound experimental approach, results that confirm or reject the hypothesis and they should offer novel insight into the existing body of knowledge. Research papers should not exceed twenty pages of printed text, including tables, figures and references (one page of printed text = approximately 600 words).

The Short communication format is intended for presentation of important observations that can be clearly described in an abbreviated format. For example, molecular data useful for typing pathogens or the first report of preliminary data would be suitable for this section.

Short descriptions of genes isolated from pathogens and pest organisms, and of plant genes with a putative function in plant– pathogen interactions can also be presented in the Short communication format. Short communications should contain firm data and will be refereed. A Short communication should have an abstract and should not exceed four printed pages in total. There are no subheadings and a description of Materials and methods should be integrated in the text.

Authors who wish to submit a Mini review should first contact the Editorial Office, since only Mini reviews on topical issues will be considered for publication. Mini reviews should not exceed 12 pages of printed text, including tables, figures, and references.

**Manuscript Submission**

*Manuscript Submission*

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

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Please follow the hyperlink "Submit online" on the right and upload all of your manuscript files following the instructions given on the screen.

Please ensure you provide all relevant editable source files. Failing to submit these source files might cause unnecessary delays in the review and production process.

*Important notes*

- Upon submission, the **e-mail addresses of all authors** will be requested. At the end of the submission process, the corresponding author will receive an acknowledgement e-

mail and all co-authors will be contacted automatically to confirm their affiliation to the submitted work.

- Please note that it is mandatory to use **line numbering** for the manuscript. If a manuscript does not have line numbers it is returned to the author.

**Title page**

*Title Page*

Please use this **template title page** for providing the following information.

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
- A clear indication and an active e-mail address of the corresponding author
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If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

*Abstract*

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

*For life science journals only (when applicable)*

Trial registration number and date of registration

Trial registration number, date of registration followed by “retrospectively registered”

*Keywords*

Please provide 4 to 6 keywords which can be used for indexing purposes.

*Declarations*

All manuscripts must contain the following sections under the heading 'Declarations'.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

*To be used for non-life science journals*

**Funding** (information that explains whether and by whom the research was supported)

**Conflicts of interest/Competing interests** (include appropriate disclosures)

**Availability of data and material** (data transparency)

**Code availability** (software application or custom code)

**Authors' contributions** (optional: please review the submission guidelines from the journal whether statements are mandatory)

*To be used for life science journals + articles with biological applications*

**Funding** (information that explains whether and by whom the research was supported)

**Conflicts of interest/Competing interests** (include appropriate disclosures)

**Ethics approval** (include appropriate approvals or waivers)

**Consent to participate** (include appropriate statements)

**Consent for publication** (include appropriate statements)

**Availability of data and material** (data transparency)

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**Authors' contributions** (optional: please review the submission guidelines from the journal whether statements are mandatory)

Please see the relevant sections in the submission guidelines for further information as well as various examples of wording. Please revise/customize the sample statements according to your own needs.

**Text**

*Text Formatting*

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

LaTeX macro package (Download zip, 188 kB)

*Headings*

Please use no more than three levels of displayed headings.

*Abbreviations*

Abbreviations should be defined at first mention and used consistently thereafter.

*Footnotes*

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation,

and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

*Acknowledgments*

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

**Scientific style**

- Please always use internationally accepted signs and symbols for units, SI units.
- Genus and species names should be in italics.

**Additional remark Terminology**

Writing plant virus name, please use the ICTV rules:

In italics only for the species name.

When refering to the virus (the thing that you can purify or that infects a plant) use the name without an capital letter and without italics.

3 examples:

….. plum pox virus (PP)….

….. carnation Italian ringspot virus (CIRV)……

….. Indian citrus ringspot virus (ICRSV)…….

How to write a virus name (Download docx, 29 kB)

## References

*Citation*

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

Ideally, the names of six authors should be given before et al. (assuming there are six or more), but names will not be deleted if more than six have been provided.

*Reference list*

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work.

Journal names and book titles should be *italicized*.

- Journal article Harris, M., Karper, E., Stacks, G., Hoffman, D., DeNiro, R., Cruz, P., et al. (2001). Writing labs and the Hollywood connection. *Journal of Film Writing*, 44(3), 213–245.
- Article by DOI Slifka, M. K., & Whitton, J. L. (2000) Clinical implications of dysregulated cytokine production. *Journal of Molecular Medicine*, https://doi.org/10.1007/s001090000086
- Book Calfee, R. C., & Valencia, R. R. (1991). *APA guide to preparing manuscripts for journal publication.* Washington, DC: American Psychological Association.

- Book chapter O'Neil, J. M., & Egan, J. (1992). Men's and women's gender role journeys: Metaphor for healing, transition, and transformation. In B. R. Wainrib (Ed.), *Gender issues across the life cycle* (pp. 107–123). New York: Springer.
- Online document Abou-Allaban, Y., Dell, M. L., Greenberg, W., Lomax, J., Peteet, J., Torres, M., & Cowell, V. (2006). Religious/spiritual commitments and psychiatric practice. Resource document. American Psychiatric Association. http://www.psych.org/edu/other_res/lib_archives/archives/200604.pdf. Accessed 25 June 2007.

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

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**Tables**

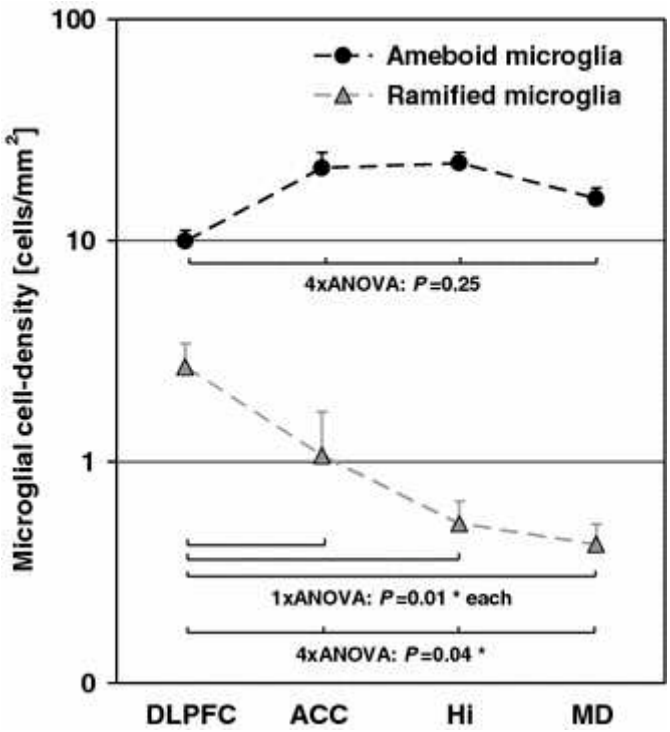
- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

**Artwork and Illustrations Guidelines**

*Electronic Figure Submission*

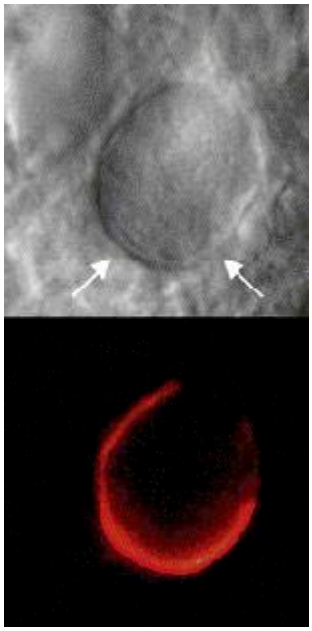
- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

*Line Art*




- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

*Halftone Art*



- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
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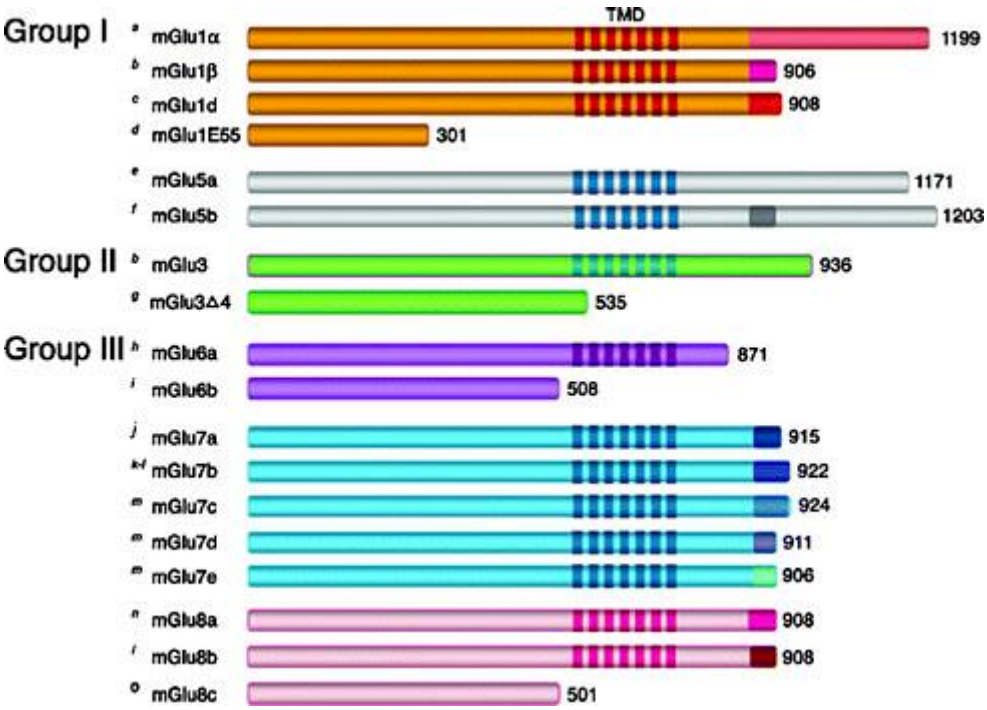

- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
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- Color art is free of charge for online publication.
- If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent.
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*Figure Lettering*

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
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