Ras2 is important for growth and pathogenicity in *Fusarium circinatum*

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Highlights

- The RAS2 gene was successfully knocked out and replaced in the genome of in *Fusarium circinatum*.
- RAS2 is important for growth in *Fusarium circinatum*.
- RAS2 is essential for pathogenicity in *Fusarium circinatum*.
- RAS2 plays a role in conidial germination in *Fusarium circinatum*.

Abstract

In this study, we investigated to possible role of Ras2 in *Fusarium circinatum*- a fungus that causes pine pitch canker disease on many different pine species and has a wide geographic distribution. This protein is encoded by the *RAS2* gene and has been shown to control growth and pathogenicity in a number of fungi in a mitogen-activated protein kinase- and/or cyclic adenosyl monophosphate pathway-dependent manner. The aim was therefore to characterize the phenotypes of *RAS2* gene knockout and complementation mutants of *F. circinatum*. These mutants were generated by transforming protoplasts of the fungus with suitable split-marker constructs. The mutant strains, together with the wild type strain, were used in growth studies as well as pathogenicity assays on *Pinus patula* seedlings. Results showed that the knockout mutant strain produced significantly smaller lesions compared to the complementation mutant and wild type strains. Growth studies also showed significantly smaller colonies and delayed conidial germination in the knockout mutant strain compared to the complement mutant and wild type strains. Interestingly, the knockout mutant strain produced more macroconidia than the wild type strain. Collectively, these results showed that Ras2 plays an important role in both growth and pathogenicity of *F. circinatum*. Future studies will seek to determine the pathway(s) through which Ras2 controls these traits in *F. circinatum*.

Keywords: Ras2; GTPase; Signaling; Pathogenicity; *Fusarium circinatum; Pinus patula*

1. Introduction

Signaling pathways that govern fungal pathogenesis and virulence have been the subject of numerous studies on important plant pathogens (Lengeler et al., 2000). This previous body of work has shown that these pathways are complex, involving many different proteins including receptors and transcription factors. The mitogen-activated protein kinase (MAPK) and cyclic adenosyl monophosphate (cAMP) pathways are among the most prominent known to be involved in fungal pathogenesis (Lengeler et al., 2000). They are activated by Ras proteins (Lengeler et al., 2000), which are not only important for the infection process, but in many cases also integral to growth and reproduction (Kronstad and Staben, 1997).

Ras proteins belong to a superfamily of small guanosine triphosphatases (GTPases) that act as molecular switches for signaling pathways in eukaryotes (Cox and Der, 2010, Barbacid, 1987). They transduce signals from external cues that culminate in the expression of genes involved in cellular functions such as growth, differentiation, metabolism and programmed cell death (Lowy and Willumsen, 1993). Overall, the Ras superfamily may be divided into five main groups based on sequence and function (i.e., Rab, Ran, Ras, Rho, and Sar/Arf), and of these the Ras group is by far the best characterized in multicellular eukaryotes (Bourne et al., 1991, Pentland et al., 2018). The Ras group proteins are encoded by *RAS* (rat sarcoma) genes, the first of which was characterized in rats where *RAS* mutations caused sarcomas due to constitutive activation of the Ras protein (Cox and Der, 2010). Because such mutations have also been linked to approximately 50% of all cancers in humans (Campbell and Der, 2004), *RAS* genes form part of many molecular diagnostic cancer panels (Sokolenko and Imyanitov, 2018).

In fungi, the two Ras proteins that have been widely studied are Ras1 and Ras2, respectively encoded by *RAS1* and *RAS2* (Toda et al., 1985, Sun et al., 1994, Mosch et al., 1996, Fillinger et al., 2002, Waugh et al., 2002, Gourlay and Ayscough, 2006, Bluhm et al., 2007, Zhu et al., 2009, Zhou et al., 2014). Depending on the fungus examined, previous work has shown that the functions of these proteins may be redundant, overlapping and/or antagonistic. These proteins can also have high sequence similarity or differ significantly in the same fungus (Zhu et al., 2009). Moreover, the motif used for membrane anchorage (i.e., CAAX where C = Cysteine; AA = any two aliphatic amino acids; X = any amino acid), and where posttranslational modifications occur for this purpose, can be the same or differ between the two proteins (Spence and Casey, 2001, Young et al., 2001, Krishnankutty et al., 2009). In *Saccharomyces cerevisiae* Ras1 and Ras2 are similar in length and CAAX motif and thus have redundant roles (Hrycyna and Clark, 1992, Zhu et al., 2009), where both proteins are capable of activating the cAMP and MAPK pathways (Toda et al., 1985, Mosch et al., 1996). Interestingly, and contrary to the case in humans, hyper-activation of the Ras2 pathway in *S. cerevisiae* leads to apoptosis (Gourlay and Ayscough, 2006). In *Aspergillus nidulans*, RasA (a homologue of Ras1) controls asexual spore germination through activation of the cAMP pathway (Fillinger et al., 2002). The same has been observed for *Neurospora crassa* (Bruno et al., 1996), *S. cerevisiae* (Thevelein, 1984) and *Schizosaccharomyces pombe* (Hatanaka and Shimoda, 2001). In *Magnaporthe oryzae*, Ras1 plays a minor role in growth, whereas Ras2 plays a more critical role in the formation of the specialized cells (appressoria) needed for initiation of plant infection (Zhou et al., 2014). In *Candida albicans*, Ras1 and Ras2 have been shown to have antagonistic roles (Uhl et al., 2003, Zhu et al., 2009), while they have overlapping and unique roles in *Cryptococcus neoformans* (Waugh et al., 2002). In the latter fungus, both proteins are involved in growth, but only Ras1 is required for its growth at high temperatures (Waugh et al., 2002).

Few studies have investigated the role of Ras1 and Ras2 in the growth, development and infection biology of *Fusarium* species. This is despite the fact that members of this genus include some of the world's most important plant pathogens (Nelson, 1981). To the best of our knowledge, a Ras protein has been investigated in only one species of the genus, i.e., *Fusarium graminearum* (Bluhm et al., 2007). In this fungus, Ras2 was shown to control virulence, growth, spore germination and perithecium formation (Bluhm et al., 2007). The study also showed that, unlike in *S. cerevisiae*, Ras2 controls these traits in *F. graminearum* through the MAPK pathway only (Bluhm et al., 2007). In the current study, we focused on the *RAS2* gene of the globally important pine pathogen *Fusarium circinatum* (Wingfield et al., 2008, Drenkhan et al., 2020). Within the broader genus, *F. circinatum* is distantly related to *F. graminearum*, with the two fungi forming part of species complexes (i.e., *F. fujikuroi* and *F. sambucinum* species complexes, respectively) that diverged more than 30 million years ago (O'Donnell et al., 2013, Geiser et al., 2020).

The whole-genome sequence for *F. circinatum* has been determined (Wingfield et al., 2012), but not much is known about the molecular processes involved in its pathogenic behavior. Although a few studies have identified genes and gene clusters potentially involved in growth and pathogenicity (De Vos et al., 2011, Wiemann et al., 2013, Van Wyk et al., 2018, Munoz-Adalia et al., 2018a), only one has functionally characterized a gene involved in the growth of *F. circinatum* (Munoz-Adalia et al., 2018b). Also, nothing is known regarding the role of Ras2 in the biology of *F. circinatum*. The aim of this study was therefore to characterize the role of Ras2 in *F. circinatum*. For this purpose, we first generated *RAS2* knockout and complement mutants using the split-marker construct protocol (Catlett et al., 2003). These mutants and the wild type strain were then used in an array of assays to assess the impact of Ras2 in growth and pathogenicity of the fungus. The findings of this study will thus increase our knowledge about the molecular basis and processes that underlie growth and pathogenicity in *F. circinatum* and likely other fungi in this genus.

2. Materials and methods

2.1. Fungal isolates, growth conditions and DNA extractions

Four *F. circinatum* isolates were used in this study. Of the four, two originated from our previous work. These were isolates KS17 and FSP34 and are available as strains CMW 674 and CMW 53647, respectively, from the culture collection of the Forestry and Agricultural Biotechnology Institute, FABI, University of Pretoria, South Africa. These two isolates are sexually compatible and can produce fertile progeny, because KS17 is female fertile with MAT2 mating specificity and FSP34 is MAT1 and largely female sterile (Desjardins et al., 2000, Steenkamp et al., 2001, Van Wyk et al., 2018). The two remaining isolates used in this study represented *ras2* knockout (ΔFcras2; CMW 53644) and complement (ΔFcras2C; CMW 53645) mutants of FSP34, which were generated in this study (see below). Fungal strains were routinely grown on half strength Potato Dextrose Agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 25 °C unless otherwise stated.

For the extraction of DNA, an agar block overgrown with mycelium was cut from a seven-day-old PDA culture and used to inoculate Potato Dextrose Broth (PDB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The PDB cultures were incubated with shaking at approximately 200 rpm for seven days at room temperature. The cultures were then filtered through two layers of Miracloth (Calbiochem, La Jolla, CA, USA) to collect fungal biomass and then freeze dried. The freeze-dried material was then used to extract DNA using the

CTAB (cetyltrimethylammonium bromide) protocol of Murray and Thompson (1980) with minor modifications (see Supplementary file S1).

2.2. Identification and analysis of *RAS* genes

To identify *RAS* genes in the genome of *F. circinatum*, the *F. graminearum RAS1* and *RAS2* gene sequences (FGSG_09778 and FGSG_10114; Bluhm et al., 2007) were used. They were retrieved from the nucleotide database of the National Center for Biotechnology Information (NCBI; https://www-ncbi-nlm-nih-gov.uplib.idm.oclc.org) using NCBI accession numbers XM_011329611.1 and XM_011320750.1, respectively. The *F. graminearum* sequences were then used as queries in BLASTn searches against the genome of *F. circinatum* FSP34 (NCBI accession number AYJV00000000; Wingfield et al., 2012) using CLC Genomics Workbench (Version 8.0.3) with default settings. The protein sequences and CAAX motifs for these genes were compared using their inferred amino acid sequences and alignment in CLC Genomics Workbench.

We also subjected the *F. circinatum RAS2* gene to phylogenetic analysis. For this purpose, the *RAS2* coding sequences from various species of *Fusarium* (including those of the *F. fujikuroi* and *F. sambucinum* species complexes) and other fungi (i.e., *Colletotrichum higginsianum, M. oryzae* (*Pyricularia oryzae*), *Trichoderma reesei*, *Alternaria burnsii*, *A. nidulans*, and *N. crassa*) were retrieved from NCBI using the Ras2 sequence of *F. circinatum* and tBLASTn searches with default parameters. We then constructed multiple sequence alignment by aligning the coding nucleotide sequences based on their corresponding amino acid translations using MAFFT (Katoh et al., 2002) in the online version of TranslatorX (Abascal et al., 2010). Following visual inspection of the alignments in AliView version 1.26 (Larsson, 2014), a maximum likelihood phylogeny was determined using raxmlGUI version 2.0 (Edler et al., 2020). This analysis utilized the TIM2 + G + I substitution model as determined with the "optimize" function in raxmlGUI. Nodal support values were calculated with bootstrap using the "thorough bootstrap" setting with 1000 replications. The tree was rooted with *Aspergillus nidulan*s and *Alternaria burnsii* as outgroup taxa.

2.3. Generation of *ras2* knockout and complementation constructs

Knockout constructs for *RAS2* gene replacement were generated using the split-marker construct protocol (Catlett et al., 2003; see Supplementary file S1). For this purpose, Polymerase Chain Reaction (PCR) amplification of the 5′ flanking region of *RAS2* was done using primer set *Ras2*U F1 + *Ras2*U R1 (Table 1), and the 3′ flank was amplified using primer set *Ras2*Dw F1 + *Ras2*Dw R1 (Table 1). We also produced two overlapping amplicons of the hygromycin resistance gene (*hygR*) by making use of two independent PCRs with primer sets HygR + ygF and HygF + hyR (Table 1; Zhou et al., 2010) that were performed directly on *Escherichia coli* colonies carrying plasmid pCB1004 (Carroll et al., 1994). All amplicons were generated using Phusion High-Fidelity Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania). The 5′ *RAS2* flank was then fused to the first *hygR* amplicon using primer set *Ras2*U F1 + ygF (Table 1; Zhou et al., 2010), and the 3′ *RAS2* flank was fused to the second *hygR* amplicon using primers *Ras2*Dw R1 and hyR. These amplicon fusion reactions were done using LongAmp® Taq 2X Master Mix (New England BioLabs, Ipswich, MA, USA).

Table 1. List of primers used in this study.[1]

Primer name	Sequence (5′ to 3′)	References
*Ras2*U F1	ATTTAAAGTCCACTGGCAGG	This study
*Ras2*U R1	ACTTATTCAGGCGTAGCAACCAGGCAGTTAAGAGGAGGAAAATGAGG	This study
*Ras2*Dw F1	TCAGCATCTTTTACTTTCACCAGCGGAAGAAAGACGACCCAAGC	This study
*Ras2*Dw R1	CTGATCCCCCTTAAAGAACC	This study
HygF	AACGCTGGTGAAAGTAAAAGATGCTG	This study
HygR	ACGCCTGGTTGCTACGCCTGAATAAGT	This study
hyR	GTATTGACCGATTCCTTGCGGTCCGAA	Zhou et al., 2010
ygF	GATGTAGGAGGGCGTGGATATGTCCT	Zhou et al., 2010
*ras2*F	TACCACACACTT	This study
*ras2*R	TTGGTGGTTCTG	This study
*ras2*_probeF1	CTGGCCGAATGGTGTTAT	This study
*ras2*_ProbeR1	GCCATCACGAATCCATTG	This study
*ras2*left_F1	CGATCTTTACTTTCCTTTCC	This study
*ras2*right_R1	TTCAGCATTCCCCATCCA	This study
Hyg-int-R	CGGCCGTCTGGACCGATGGCTGTGTA	This study
*ras2*allele_F1	TACTCACCTGACACGACTC	This study
*ras2*allele_R	CTCCAAATAATAGCCACAAC	This study
Not_1F_rp619	CTGTTGTGGCTATTATTTGGAGCATGCGGCCGCATGCCAGTTG	This study
Not_1R_rp620	CATGCGGCCGCAGAGTAAAG	This study
TrpC_Probe_F	CAGATATAGTTCCCGGAG	This study
TrpC_Probe_R	GGATTGATGAAATCAACG	This study
CIRC1A	CTTGGCTCGAGAAGGG	Schweigkofler et al., 2004
CIRC4A	ACCTACCCTACACCTCTCACT	Schweigkofler et al., 2004

[1]See Supplementary file S1 for PCR cycling conditions employed for all primer pairs utilized in this study.

A complementation construct of the *RAS2* gene was generated for random integration into the genome of the ΔFcras2 mutant. This was done by amplifying the FSP34 *RAS2* allele (the full gene sequence including the promoter and terminator regions) using Expand™ Long Range dNTPack (Sigma-Aldrich, Mannheim, Germany) with the primers *ras2*allele_F1 and *ras2*allele_R (Table 1; Supplementary file S1). This amplicon was then fused to a geneticin resistance cassette (i.e., *nptII*) which included a promoter and terminator. The cassette was amplified directly from *E. coli* colonies carrying plasmid pGEN-Not1 (Proctor et al., 2008) using Phusion High-Fidelity Master Mix and the primer set Not_1F_rp619 + Not_1R_rp620 (Table 1). Fusion of the *RAS2* allele to the geneticin resistance cassette was performed using LongAmp® Taq 2X Master Mix and the primers *ras2*allele_F1 and Not_1R_rp620.

2.4. Transformation of wild type and mutant strains

Protoplasts of *F. circinatum* FSP34 were generated using the protocol for *F. graminearum* (Hallen-Adams et al., 2011; Supplementary file S1). About 0.25–0.5 µg of the respective

split-marker knockout constructs generated were incubated with *F. circinatum* FSP34 protoplasts in 9 ml of regeneration liquid medium with shaking at 75 rpm for about 18 h. This mixture was then poured onto 90 mm Petri dishes (1 ml per plate) and mixed with 10 ml of regeneration agar medium containing 150 µg/ml of Hygromycin B solution from *Streptomyces hygroscopicus* (Sigma-Aldrich, Mannheim, Germany). Plates were incubated in the dark at room temperature for 14 days.

Screening for potential mutants was done by transferring grown colonies onto PDA medium containing 150 µg/ml of Hygromycin B and incubating them for seven days in the dark (Supplementary file S1). We then subjected putative transformants to PCR with primer sets *ras*2F + *ras*2R and HygF + HygR to confirm the presence of the *RAS2* and *hygR* genes, respectively (Table 1). To test for the replacement of *RAS2* by the *hygR* gene in mutants, PCR was done using primers that are complementary to the flanking regions of the 5′ and 3′ *ras2* flanks (i.e., *ras2*left_F1 and *ras2*right_R1; see Table 1) by employing the LongAmp® *Taq* 2X Master Mix. To determine the copy number of the *hygR* construct in genomes of the mutants, a Southern Blot analysis was done using a protocol adapted from Sambrook and Green, 2012, Eisel et al., 2008 (See Supplementary file S1). The primer set HygR + Hyg-int-R (Table 1) were used to synthesize the probe used during the Southern Blot analysis.

In order to produce complementation mutants, protoplasts were generated from ΔFcras2 and the protoplasts were transformed with the complementation construct as described above. However, the overnight cultures were mixed with regeneration agar medium containing 120 µg/ml of geneticin (G-418 sulfate; Thermo Fisher Scientific, Rockford, IL, USA). Further culturing of colonies was done on PDA augmented with 120 µg/ml of geneticin (G-418 sulfate) and incubated in the dark for seven days. To test for the presence of the *RAS2* gene in complementation transformants, colony PCR amplification was performed using *Ras2*_probeF1 and *Ras2*_probeR1 (Table 1) with the LongAmp® *Taq* 2X Master Mix.

2.5. Morphological traits of mutants and wild type strains

General colony morphology of the FSP34 wild type and the knockout and complement mutant strains were examined. After seven days of growth on Spezieller Nährstoffarmer Agar (SNA; Leslie and Summerell, 2006), agar discs overgrown with mycelium were punched out of each culture using a sterile hollow glass rod with 5 mm diameter. These discs were each placed onto 90 mm Petri dish plates containing PDA and incubated at 24 °C in i) darkness, ii) under continuous light, and iii) under a 12/12-hour light/dark cycle using near UV light. For assessments of fungal micromorphological structures, fungi were grown on Carnation Leaf Agar (CLA; Fisher et al., 1982) and SNA with sterile carnation leaves, and incubated at room temperature (22–24 °C) under a 12/12-hour near-UV light/dark cycle. Microscopic observations were made under a light microscope using a standard slide and water as a mounting medium. Photographs were captured at 63x magnification.

Colony growth of the FSP34 wild type and the knockout and complement mutant strains was measured. For this, 50 000 spores/ml spore suspensions were prepared by flushing 4 ml of sterile distilled water onto 7-day-old PDA cultures and enumerating the spores using a hemocytometer slide and 40x magnification under a light microscope. For each of the respective strains, 10 µl of spore suspension were used to inoculate PDA medium. Following incubation at 25 °C for seven days, agar discs overgrown with mycelium were punched out of each culture using a sterile hollow glass rod with 5 mm diameter. These discs were each placed onto the center of 90 mm plates containing PDA and incubated under the following

conditions: i) 25 °C under light, ii) 25 °C in darkness, iii) 15 °C under light and iv) 15 °C in darkness. Six replicates were used for each of the culturing conditions and perpendicular colony diameter measurements were taken after seven days. Similar sets of experiments were also used to evaluate growth rate, where inoculated plates were incubated for seven days and colony diameters recorded daily from days four to seven.

The number of conidia produced by the wild type and the knockout and complement mutant strains were examined. Conidial suspensions were prepared using 5 mm diameter discs overgrown with mycelium from ten-day-old PDA cultures incubated at 25 °C under light. These discs were placed in 2 ml tubes containing 1.5 ml sterile distilled water, and then agitated with a vortex mixer for 30 s to release spores into the water. The number of spores in 10 µl of each suspension was then quantified using a hemocytometer slide and 40x magnification under a light microscope. For these spore suspensions, the relative abundance of microconidia and macroconidia (the latter included mesoconidia) were also determined. This was done by placing 10 µl of the respective suspensions on standard microscope slides and counting the two conidial types using a light microscope. In all of these cases, three replicates were examined per strain.

Conidial germination was assessed for each of the wild type and the knockout and complement mutant strains. To this end, conidial suspensions were prepared by flushing 4 ml of sterile distilled water onto 10-day-old PDA cultures (incubated at 25 °C under light) of each strain. One milliliter of each suspension was then pipetted into sterile 12-well cell culture plates, which were then incubated with shaking (80 rpm) at 23 °C. Three replicate assessments were made for each strain. Following 24, 48 and 72 h, 10 µl of the respective spore suspensions were collected and assessed under a light microscope by measuring the length of the hyphal strand that emerged from conidia of each strain during germination.

2.6. Fertility of wild type and mutants and sexual crosses

Sexual fertility of the FSP34 wild type and the knockout and complement mutant strains with *F. circinatum* KS17 was assessed. Because previous experiments have shown that FSP34 is predominantly female sterile (Desjardins et al., 2000), the assessment focused on examination of the male fertility of FSP34 and its mutants. This was done by crossing each of the mutant strains and FSP34 with the female fertile isolate KS17. Accordingly, we cultivated KS17 on Carrot Agar (Leslie and Summerell, 2006) and the respective FSP34 wild type and mutant strains on Complete Medium (Leslie and Summerell, 2006) for seven days in the dark at room temperature. Spores produced by the Complete Medium cultures were then suspended in a 2.5% Tween 20 solution (Sigma-Aldrich, Mannheim, Germany), of which about 1 ml was applied across the surface of a KS17 culture with a cell spreader. Perithecium formation was evaluated after six weeks following incubation at ±17 °C under near-UV light (Leslie and Summerell, 2006). The crosses were replicated four times for each of the FSP34 wild type and mutant strains.

2.7. Pathogenicity of wild type and mutants on pine seedlings

Pathogenicity of the FSP34 wild type and the knockout and complement mutant strains was tested on six-month-old *P. patula* seedlings. Conidial suspensions (50 000 conidia/ml) were prepared following the same procedure used for the growth study. Stem tips were removed five centimeters from the growth tip of the seedlings. To the cut plant surface, we then applied 10 µl of the prepared conidial suspension, while 10 µl of a 15% glycerol solution was

used as control. Each strain was used to inoculate forty *P. patula* seedlings and the same was also true for the control treatment. Inoculations were done in a randomized manner and the randomization layout design was done in Microsoft® Excel using the RAND function. Seedlings were watered once every day for six weeks after which lesion lengths (mm) were measured.

To meet the requirement of Koch's postulates, isolations were made from lesions on the inoculated seedlings. This was done by cutting diseased and non-diseased stem tips, removing the needles, and placing the stem tips into 90 mm Petri dishes containing *Fusarium* selective medium (FSM) (Leslie and Summerell, 2006). Following incubation for seven days at 25 °C in the dark, DNA was extracted (as described earlier) from the fungi growing on the FSM plates. These DNAs were then used to confirm that they represent *F. circinatum* using the diagnostic PCR protocol that uses *F. circinatum* species-specific primer set CIRC1A + CIRC4A (Table 1; Schweigkofler et al., 2004, Steenkamp et al., 2014) and *Taq* DNA polymerase (Roche Diagnostics, Mannheim Germany). We also confirmed presence/absence of the *RAS2* gene in these isolates using PCR with the primer set *ras2*_ProbeF1 + *ras2*_ProbeR1 (Table 1). The presence/absence of the hygromycin resistance gene was also confirmed, using primers hy-R and HygF (Table 1), and the LongAmp® *Taq* 2X Master Mix.

2.8. Statistical analyses

To test for variation in lesion lengths between infected seedlings as well as variation in culture growth and conidial production in the FSP34 wild type and the knockout and complement mutant strains, analysis of variance (ANOVA) was used (http://astatsa.com/OneWay_Anova_with_TukeyHSD/). The null (H_o) hypothesis was tested using the F test where H_o was that all the (mean) lesion lengths produced by the different strains or their growth rates are equal (Samuels and Witmer, 2003). For this purpose, the F statistic was compared to an F distribution critical value at a 95% confidence level (P = probability = 0.05) (Samuels and Witmer, 2003). Tukey's Honestly Significant Difference (HSD) test was used to test which of the treatments were significantly different from each other. Tukey's statistic value equation q

was used. In this equation, q is Tukey's statistic, M_1 and M_2 are the two means being tested, MS_w is the mean of squares within the data, and n is the number of samples per treatment group.

3. Results

3.1. Identification and analysis of *RAS* genes

The *RAS1* and *RAS2* genes were identified in the genome of *F. circinatum* wild type strain FSP34 using a BLASTn search with the *F. graminearum RAS1* and *RAS2* gene sequences as a query. This resulted in a hit to FCIRG_05034 and FCIRG_08573 (nucleotide sequence data for this gene are available in the Third Party Annotation Section of the DDBJ/ENA/GenBank databases under the accession number TPA: BK014288), respectively. The *F. circinatum RAS1* gene had 79% identity to that of *F. graminearum* (Expect-value = 0.0). The *RAS1* gene of *F. graminearum* is 892 base pairs (bp) in length, while *F. circinatum RAS1* is 891 bp long. Both genes have three introns at the same positions. The *F. circinatum RAS2* gene had 85% identity to that of *F. graminearum* (i.e., FGSG_10114; Expect-value = 0.0). The *RAS2* gene

of *F. graminearum* is 708 base pairs (bp) in length, while its ortholog in *F. circinatum* is 773 bp long. This is due to a 65-bp intron in *RAS2* of *F. circinatum* compared to the *RAS2* of *F. graminearum*, which does not have that intron.

The *F. circinatum* and *F. graminearum RAS2* genes both coded for a protein containing 235 amino acids. Also, they shared 98.7% sequence similarity (differing with only 3 amino acids) and had the same CAAX motif (i.e., CVVL) at their C-terminals. The inferred Ras2 protein sequence further showed conservation (including in the CAAX motif) when compared with that of other *Fusarium* species and fungi such as *C. higginsianum*, *M. oryzae*, *T. reesei*, *A. burnsii*, *A. nidulans*, and *N. crassa* (not shown). However, comparison of the Ras2 protein sequence of *F. circinatum* with its Ras1 sequence showed only 50.2% similarity, while the *F. circinatum* and *F. graminearum* Ras1 protein sequences were highly similar (i.e., both consisting of 217 amino acids, at an identity of 99.5%, and with the CAAX motif of CVLM at the C-terminal). Phylogenetic analysis of the *RAS2* coding sequences clustered *F. circinatum* in a well-supported clade containing other representatives of the *F. fujikuroi* species complex and the closely related *F. oxysporum* species complex, while *F. graminearum* formed part of a separate clade containing other representatives of the *F. sambucinum* species complex (Geiser et al., 2020) (Supplementary figure S1). Within the *RAS2* phylogeny, these two species complexes formed sister clades, with a representative of the *F. solani* species complex at their base (Geiser et al., 2020, O'Donnell et al., 2020) (Supplementary figure S1).

3.2. Knockout and complementation of *RAS2*

The *RAS2* knockout experiment in *F. circinatum* yielded 13 potential transformants that grew on PDA supplemented with hygromycin B. Polymerase Chain Reaction experiments showed that *hygR* was present in nine of these, and in eight of these, *RAS2* was absent. Southern blot experiments showed the integration of only one copy of *hygR* into the genome of some of the latter eight isolates. Of the single-copy *hygR* mutant strains, one (ΔFcras2) was randomly selected for further phenotypic studies. Our PCR assays showed that *RAS2* had been replaced with *hygR* in the genome of ΔFcras2. Complementation of ΔFcras2 with the *RAS2* allele resulted in more than 20 colonies growing on PDA amended with geneticin, although only two of them contained the *RAS2* gene. One of these complements (ΔFcras2C) was selected for use in further phenotypic studies. In other words, all further studies considering the function of *RAS2* were conducted with strains ΔFcras2, ΔFcras2C and the wild-type strain FSP34.

3.3. *RAS2* gene deletion affects the morphology and growth of *F. circinatum*

Overall morphological assessments showed differences between the knockout mutant strain ΔFcras2, the complement mutant ΔFcras2C and wild type strains FSP34 (Fig. 1). Colonies of the knockout mutant strain appeared smaller than those of the wild type and complement strains (Fig. 1A). The wild type and complement mutant strains produced sterile coils, while the knockout mutant strain ΔFcras2 did not (Fig. 1B). Aerial conidia of FSP34 were predominantly produced by monophialides and those of the complement and knockout mutant strains were produced by mono and polyphialides (Fig. 1B). All strains produced both micro- and macroconidia, but ΔFcras2 also produced mesoconidia, which were not observed in the other two strains (Fig. 1B).

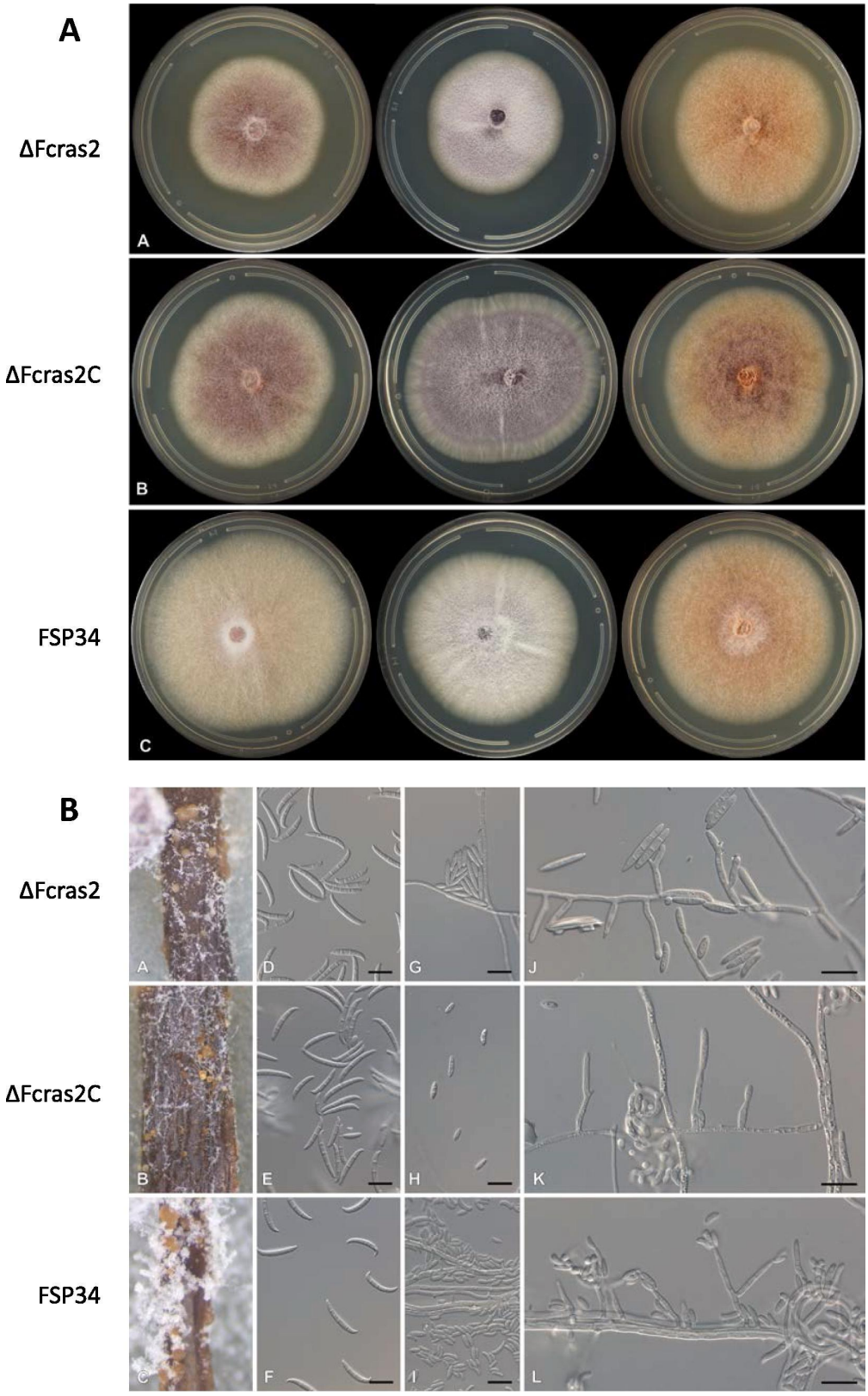

Fig. 1. A. Colony morphology of the Knockout mutant strain ΔFcras2 (A), Complement mutant strain ΔFcras2C (B) and Wild type strain FSP34 (C) on PDA after 7 days of incubation at 24 °C in the dark, light and near-UV light, respectively. **B.** Sporodochia of ΔFcras2, ΔFcras2C and FSP34 on carnation leaves, A–C respectively. D–F: Sporodochial conidia (macroconidia) of ΔFcras2, ΔFcras2C and FSP34, respectively. G-I: Aerial conidia for of ΔFcras2, ΔFcras2C and FSP34, respectively. J-L: Aerial conidiophores for of ΔFcras2, ΔFcras2C and FSP34, respectively.

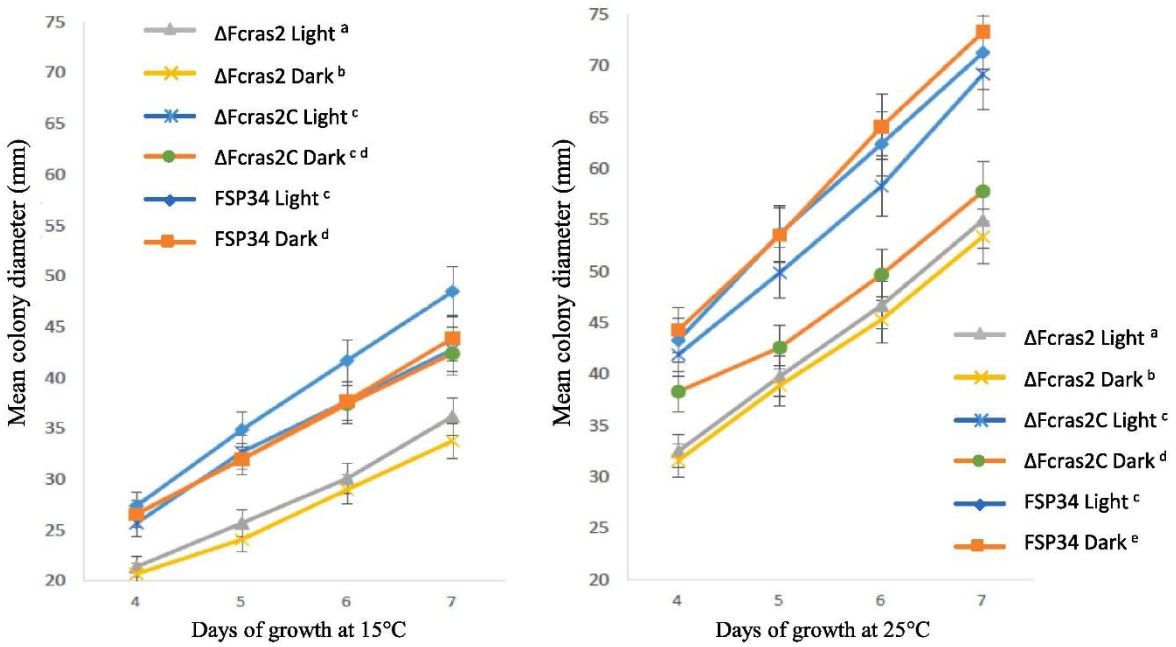

Fig. 2. Daily (day four to seven) growth of ΔFcras2, ΔFcras2C and FSP34 at 15 °C and 25 °C under light and dark conditions. Statistical significance between the three strains was tested at $P \leq 0.05$ using six replicates per strain. Strains with different letters show significant difference, while those with the same letter were not significantly different.

Table 2. Average colony diameter of the three *F. circinatum* strains after seven days of incubation at 25 °C and 15 °C on PDA medium under light and darkness.

Strain	Incubation temperature	Colony diameter (mm) of week-old cultures[1]		Statistical difference in growth under light vs darkness[2]
		Light	Dark	
ΔFcras2	25°	57.3 (0.63)*	53.3 (0.58)*	Significant
ΔFcras2C		69.2 (0.76)	57.8 (0.58)*	Significant
FSP34		71.3 (0.27)	73.3 (0.36)	Significant
ΔFcras2	15 °C	36.2 (0.34)*	33.7 (0.39)*	Significant
ΔFcras2C		42.8 (0.43)	42.4 (0.60)	Not significant
FSP34		48.5 (0.26)	43.9 (0.27)	Significant

[1]Values are the average for six replicates per strain, and the Standard Error (expressed in mm) values are shown in brackets. Asterisks indicate values that differ statistically ($P \leq 0.05$) from that of the wild type strain (FSP34) under a specific set of growth conditions.
[2]Statistical significance was tested ($P \leq 0.05$) between growth in light and darkness, using six replicates for each strain at each temperature.

The growth of the knockout mutant strain ΔFcras2 was significantly reduced compared to that of the wild type strain FSP34 and complement knockout strain ΔFcras2C (Fig. 2, Table 2). These results were observed following incubation at 25 °C and at 15 °C in both light and darkness. In these experiments, the wild type strain FSP34 always formed the largest colonies. When comparing growth of the mutants under light and darkness at these temperatures, ΔFcras2 and ΔFcras2C always grew faster in the light than in the dark, with significant differences detected for all comparisons except when ΔFcras2C was grown at 15 °C. The wild type strain also grew more rapidly under light than in darkness at 15 °C, but

at 25 °C it grew significantly faster in light. Additionally, the shape of the colonies produced by the knockout mutant strain ΔFcras2 were mostly asymmetrical whereas those of the wild type strain FSP34 were more symmetrical at 25 °C (Fig. 3). This was evident from the differences between perpendicular colony diameter measurements that were substantially smaller in the case of FSP34 than for Fcras2 and Fcras2C. At 15 °C these differences were much less pronounced, but colonies of the two mutant strains were also more asymmetrical than those of the wild type strain (not shown).

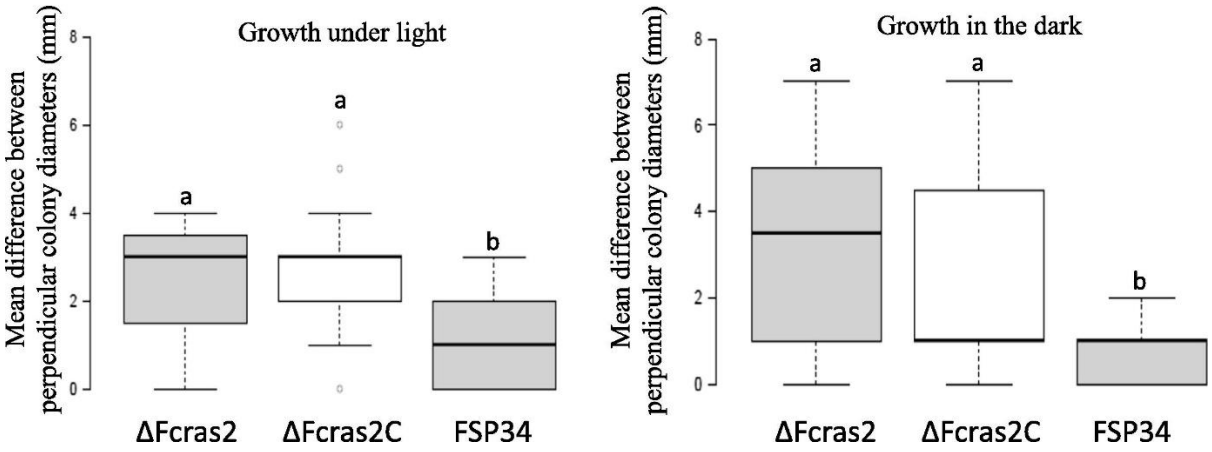

Fig. 3. Symmetry of ΔFcras2, ΔFcras2C and FSP34 colonies under light and in the dark at 25 °C. Bottom and top whiskers represent the 1st and 4th quartiles (Q1 and Q4) of the data respectively. Points outside whiskers represent outliers. Boxes bound the interquartile range (Q2 and Q3) and the solid line in boxes show the median. Wider boxes illustrate asymmetrical colonies whereas narrow and clear boxes display symmetrical colonies. Statistical significance was tested at $P \leq 0.05$ using six replicates per strain. Boxplots with different letters show significant difference, while those with the same letter were not significantly different. Boxplots were constructed using the online tool at http://shiny.chemgrid.org/boxplotr/.

3.4. Conidium production and germination are affected by *RAS2* gene deletion

The deletion of *RAS2* resulted in significant differences between wild type and mutant strains with regards to the production of conidia (Table 3). On average, the knockout mutant ΔFcras2 produced more conidia than either the complement mutant (ΔFcras2C) or the wild type strain (FSP34). However, for both mutant strains, most of these (44–49%) were represented by macroconidia. By contrast, macroconidia represented only about 3% of the conidia produced by the wild type strain (Table 3).

Table 3. Conidial production by the *F. circinatum* wild type and mutant strains after ten days of incubation at 25 °C, under light on PDA medium.

Strain	Total number of micro- and macroconidia produced ($\times 10^6$ spores/ml)[1]	Proportion of macroconidia produced (%)[2]
ΔFcras2	8.48 (0.52) *	48.54 (2.52) *
ΔFcras2C	5.35 (0.59) *	44.74 (3.95) *
FSP34	3.20 (0.47)	3.25 (1.15)

[1]Values are the average for three replicates per strain, and the Standard Error (expressed as a factor of 10^6 spores/ml) values are shown in brackets. Asterisks indicate values that differ statistically ($P \leq 0.05$) from that of the wild type strain, FSP34.

[2]Values represent the average for three replicates per strain. The Standard Error, expressed as percentages, values are shown in brackets. Asterisks show percentages that differ statistically ($P \leq 0.05$) from that of the wild type strain (FSP34).

Examination of conidial germination further showed that germination of conidia was delayed in ΔFcras2 compared to ΔFcras2C and FSP34. This pattern was observed at all time points investigated (24, 48 and 72 h), although very little conidial germination occurred prior to 24 h in strain ΔFcras2. Germination of conidia produced by ΔFcras2 resulted in significantly shorter hyphae or germination tubes than in the case of FSP34. However, no differences were observed in the germ tube lengths for the conidia of ΔFcras2C and FSP34 (Table 4). We did not observe notable differences in the amount of germinating conidia in the three strains (data not shown).

Table 4. Germination of *F. circinatum* strains at the 24, 48 and 72-hour time points.

Strain	**Germ tube length (µm)** [1]		
	24 h	**48 h**	**72 h**
ΔFcras2	7.7 (0.95) *	24.7 (9.06)*	25.6 (4.35)*
ΔFcras2C	44.3 (6.39)	125.7 (2.85)	184.3 (31.49)
FSP34	86.6 (20.83)	96.0 (6.85)	133.1 (0.95)

[1]Values are the mean length of the germ tubes/hyphae that emerged from conidia for three replicates per strain, and the Standard Error (expressed in µm) values are shown in brackets. Asterisks indicate values that differ statistically ($P \leq 0.05$) from that of the wild type strain, FSP34.

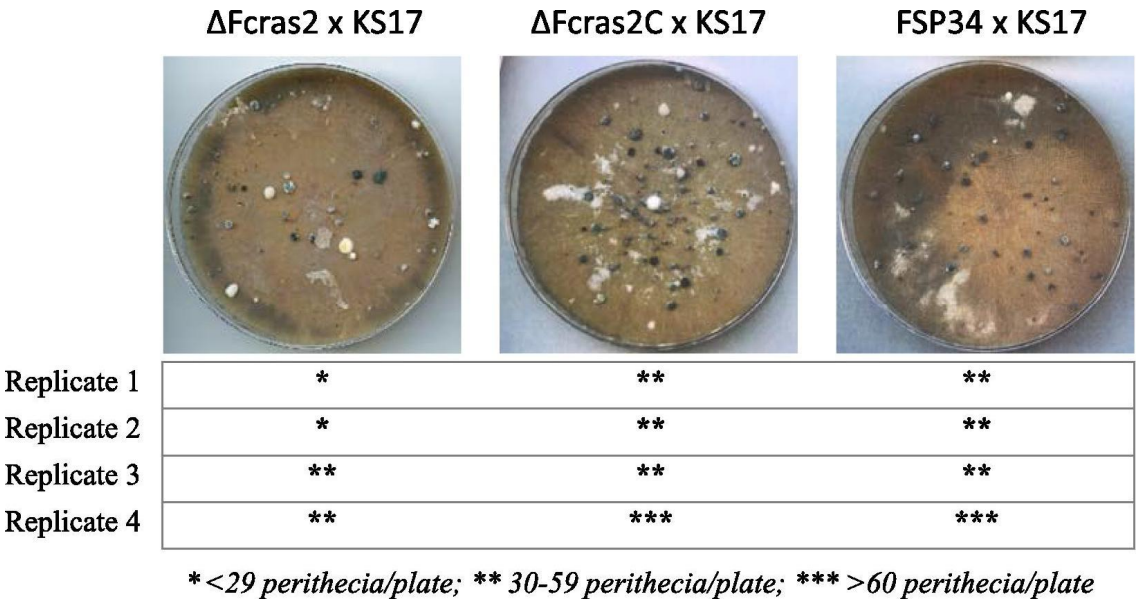

Fig. 4. Perithecial production in ΔFcras2, ΔFcras2C and FSP34 on carrot agar. Isolate KS17 was used as a female for these crosses. The crosses were replicated four times for each strain, and these are shown as replicate one to four in the figure. The symbol + is used to indicate the amount of perithecia produced in each cross (+indicates 0–29 perithecia. ++ indicates 30–59 perithecia. +++ indicates 60–89 perithecia).

3.5. Deletion of RAS2 reduces perithecial production

To determine whether *RAS2* deletion potentially impacts sexual reproduction in *F. circinatum*, a number of sexual crosses were performed by mating FSP34 and mutant strains with the female fertile MAT2 strain, KS17. Our results showed that the knockout mutant Fcras2 was generally less fertile as a male than the wild type in these crosses (Fig. 4). In other words, mating of KS17 as the female parent with ΔFcras2 as the male parent generally

resulted in the formation of substantially fewer perithecia (i.e., 12–45 perithecia per plate) than when FSP34 was used as the male parent (i.e., 32–73 perithecia per plate). However, large variations were observed between each set of four replicate plates, which precluded detection of statistically significant differences.

3.6. RAS2 gene deletion affects the pathogenicity of F. circinatum

Six weeks post-inoculation, stems and needles of diseased seedlings inoculated with FSP34 and its mutants were discolored, wilted, and necrotic, while the set of seedlings in the control treatment appeared healthy (Fig. 5A). Polymerase Chain Reaction analysis using the species-specific primer set CIRC1A + CIRC4A and DNAs extracted from isolates obtained from the diseased seedling tissue confirmed that the symptoms were caused by *F. circinatum*. Also, presence of amplicons of approximately 300 bp that were generated with primers *ras2*_ProbeF1 + *ras2*_ProbeR1 confirmed presence of the *RAS2* gene only in strains FSP34 and ΔFcras2C. Finally, amplicons of approximately 1000 bp that were generated with primers hy-R and HygF confirmed presence of the hygromycin resistance cassette only in strains ΔFcras2 and ΔFcras2C. These tests thus proved that the differences observed in disease severity between treatments were due to the presence/absence of the *RAS2* gene in the different inoculum strains.

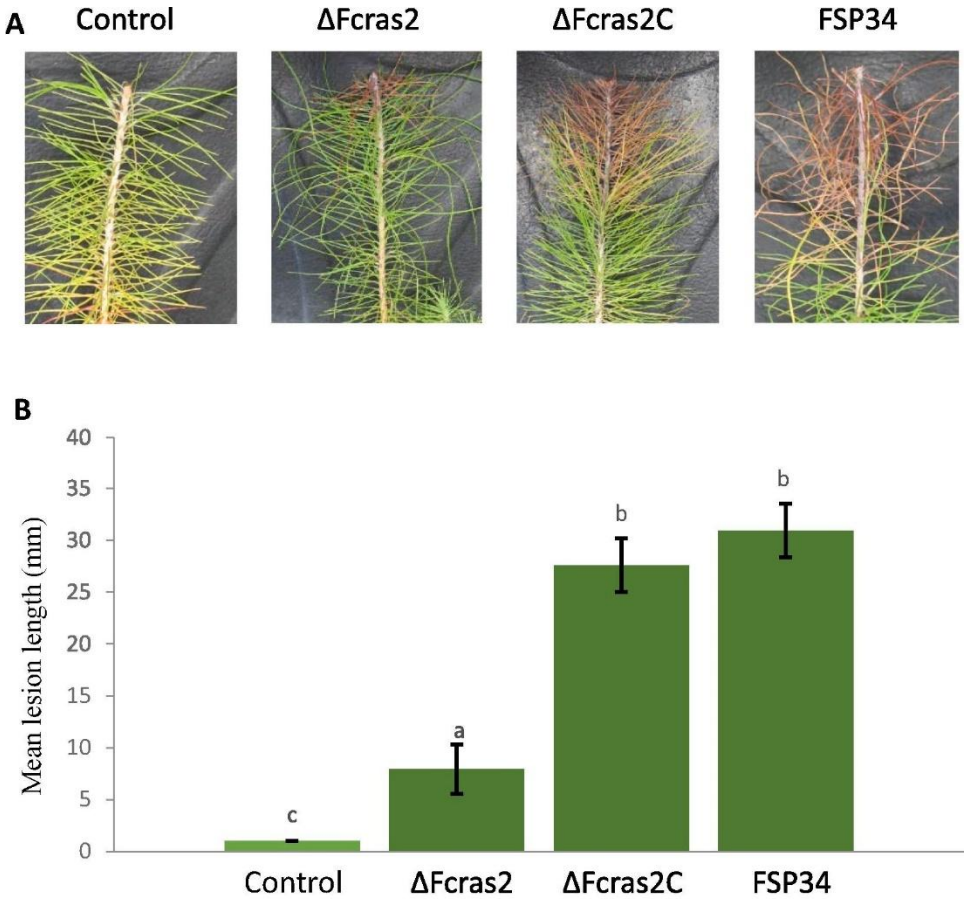

Fig. 5. The pathogenicity of ΔFcras2, FSP34 and ΔFcras2C on *P. patula*. **A.** Lesions produced on *P. patula* seedlings after inoculation with 15% glycerol, ΔFcras2, FSP34 and ΔFcras2C. **B.** Mean lesion lengths caused by inoculations of *P. patula* with 15% glycerol, ΔFcras2, ΔFcras2C and FSP34. Inoculations were performed on forty seedlings per strain, and statistical significance was tested at $P \leq 0.05$. Means with different error bar letters are significantly different from each other, and those with the same letters are not significantly different from each other.

The lesions caused by FSP34 and the two mutants were all significantly longer than the 1 mm lesions obtained for the control treatment (i.e., inoculation of seedlings with 15% glycerol) (Fig. 5B). However, the wild type strain (FSP34) produced significantly longer lesions on the pine seedlings than the knockout mutant ΔFcras2. The lesions induced by ΔFcras2 were on average 8 mm in length, while those caused by FSP34 had an average length of 31 mm. The latter were not significantly different from those produced by the complement strain ΔFcras2C.

4. Discussion

Our findings demonstrated that Ras2 plays an important role in the growth and pathogenicity of *F. circinatum*. The *RAS2* knockout mutant strain caused significantly smaller lesions on *P. patula* seedlings than the wild type and complementation mutant strains. The knockout mutant also grew significantly slower than the wild type and complementation mutant. Such reductions in virulence and growth rate, following deletion of *RAS2*, have also been reported for *F. graminearum* (Bluhm et al., 2007) and *U. maydis* (Lee and Kronstad, 2002). These mutant phenotypes are likely due to Ras2 not being available to relay crucial external stimuli (Pentland et al., 2018), which in turn impedes the activation of transcription factors responsible for the activation of genes involved in growth and virulence. In wild type cells, Ras proteins regulate cellular activities by cycling between GDP-bound and GTP-bound forms, where the GTP-bound Ras protein is active and the GDP-bound form is inactive (Boguski and McCormick, 1993). In *F. graminearum* and *U. maydis*, Ras2 has been shown to regulate virulence and pathogenicity in a MAPK pathway-dependent manner (Lee and Kronstad, 2002, Bluhm et al., 2007). In these fungi and in *C. albicans*, growth is controlled in a similar manner (Lengeler et al., 2000, Lee and Kronstad, 2002, Bluhm et al., 2007). It is therefore likely that Ras2 impacts growth and pathogenicity in *F. circinatum* in a similar manner.

In *F. circinatum*, Ras2 appears to affect colony development under light and dark conditions. In contrast to the wild type that produced larger colonies in the dark than under light, both the *ras2* knockout and complementation mutants formed larger colonies when grown under light. Other *Fusarium* species have also been reported to have enhanced growth in the dark. These include *F. oxysporum*, *F. sacchari*, *F. globosum*, *F. proliferatum* and *F. solani* (Mohsen et al., 2016). Likewise, fungi such as the black mold *Stachybotrys chartarum*, a grain pathogen, have also been shown to grow optimally in the dark (Curran, 1980). This has led to the hypothesis that some phytopathogenic fungi are adapted to grow optimally in darker environments because of the limited illumination in the plant tissues that they infect (Isaac, 1995). This could explain why the wild type of *F. circinatum* grows more rapidly in the dark, but there is no plausible molecular explanation for the increased growth exhibited by the *ras2* knockout mutant. Our results do, however, show that Ras2 plays an important role in mycelial growth of *F. circinatum* in the dark. The fact that the complementation mutant (ΔFcras2C) did not regain its ability to grow optimally in the dark is likely because the *RAS2* gene was randomly integrated into the knockout mutant's genome (and not in the same position as it is in the wild type strain). This positioning of the gene outside of its appropriate regulatory network could potentially explain why the phenotype of the complement strain was not restored fully. However, using our unpublished transcriptome data, we found no significant change in *RAS2* expression between the complement mutant and wild type strains, suggesting that the exact mechanism underlying partial restoration of some phenotypes of the complement mutant requires further exploration.

Deletion of *RAS2* impacts the shape of colonies produced by *F. circinatum* when it is grown on agar medium. Colonies produced by the wild type strain were symmetrically circular, while those produced by the knockout mutant were asymmetrical. A similar observation has been made for *F. oxysporum* following the disruption of *fga1* that encodes the alpha subunit of a G-protein (guanine nucleotide-binding protein) (Jain et al., 2002). G-proteins are GTPases and, together with septins, have been found to affect overall colony shape by regulating cell shape as seen in *U. maydis* (Becht et al., 2006, Mahlert et al., 2006). During vegetative growth and membrane expansion, cell wall deposition is mainly confined to hyphal tips, giving hyphae their elongated shape (Harris, 2012). When an entire network of these hyphae comes together, colonies that are symmetrical and evenly shaped are produced (Harris, 2012). Therefore, Ras2 (also a G protein) may be involved in the regulation of hyphal branching and colony shape in *F. circinatum*. Given that the most symmetrical colonies of the wild type and the most asymmetrical colonies in the *ras2* knockout were observed in the dark, light (or absence thereof) is likely a key stimulus for this regulation.

Deletion of *RAS2* did not cause a reduction in the number of conidia produced by *F. circinatum*, which was in contrast to what has been reported for *N. crassa* (Kana-uchi et al., 1997). Compared to the wild type and complementation mutant, the *F. circinatum ras2* knockout mutant produced significantly more conidia. However, this greater number of conidia was largely attributed to production of macroconida (and mesoconidia), because macroconidium production on PDA is exceedingly rare in *F. circinatum* and many other *Fusarium* species (Leslie and Summerell, 2006). Thus, deletion of *RAS2* enhanced macroconidium production in *F. circinatum*, as these spores represented almost 50% of the conidia produced by the *ras2* knockout mutant. This is contrary to what has been found in *F. graminearum* where deletion of *RAS2* had no negative impact on the production of macroconidia (Bluhm et al., 2007). In a study on *F. verticillioides*, deletion of *Fvmk1* (encoding a MAPK protein) resulted in an 11% increase in macroconidium production relative to the wild type (Zhang et al., 2011). To the best of our knowledge, the present study is the first to report that deletion of *RAS2* in *Fusarium* causes an increase in the production of macroconidia. Consequently, our results suggest that, like *Fvmk1* in *F. verticillioides*, Ras2 negatively regulates macroconidium production in *F. circinatum*. It would be interesting to understand whether this is also in a MAPK pathway-dependent manner, especially given that the cAMP pathway positively regulates macroconidium production in *F. verticillioides* (Zhang et al., 2011).

Similar to observations in *A. fumigatus* and *F. graminearum*, deletion of *RAS2* in *F. circinatum* significantly impacted conidium germination (Fortwendel, 2005, Bluhm et al., 2007). This was evident from the fact that the conidia produced by the *ras2* knockout mutant of *F. circinatum* had significantly shorter germ tubes than the wild type and the complementation mutant. In *A. fumigatus*, deletion of the *RASB* gene (a homologue of *RAS2*) resulted in decreased conidial germination (Fortwendel, 2005). In *F. graminearum*, deletion of *RAS2* caused conidia to germinate much more slowly than those of the wild type strain (Bluhm et al., 2007). Together with the other shared phenotypes between *ras2* knockout mutants of *F. circinatum* and *F. graminearum*, these findings show that the function *RAS2* has been maintained across 30 million years of evolution (Geiser et al., 2020). Furthermore, conidium production, viability and germination rate represent possibly important fitness traits that are often important during plant infection and disease establishment (Pringle and Taylor, 2002, Agrios, 2005). Elucidation of how *RAS2* regulates these properties in *F. circinatum* would thus be invaluable to ultimately understand and potentially manipulate the molecular ecology of this fungus.

Results of this study suggest that Ras2 is required for male fertility in *F. circinatum*. Sexual crosses involving the *ras2* knockout mutant as a male partner to a compatible female partner, generally led to the production of fewer perithecia production than when the wild type was used as a male partner. This is similar to previous observations with *ras2* deletion mutants of *F. graminearum* and *U. maydis*. In *U. maydis*, *ras2* mutants were defective in pheromone production, which negatively affected mating (Lee and Kronstad, 2002). In *F. graminearum*, Ras2 regulated perithecium production in a MAPK pathway-dependent manner (Bluhm et al., 2007). This was because deletion of the *RAS2* gene in that fungus impaired the activation of Gpmk1, thereby negatively affecting perithecia production (Bluhm et al., 2007). It would be interesting to examine the extent to which *RAS2* affects the overall process of sexual reproduction in *F. circinatum*. Crosses with the current *ras2* knockout mutant to a *ras2* knockout mutant of the female partner could expand our understanding of the role of Ras2 in the mating system of this fungus.

This study has shown that the deletion of the *RAS2* gene impairs the growth and development of *F. circinatum* as well as its virulence on *P. patula* seedlings. We were also able to demonstrate its role in macroconidium production, and apparently perithecia formation. These data are consistent with other functional characterization studies that have shown that the deletion of *RAS2* leads to developmental defects in some fungi (Lee and Kronstad, 2002, Fortwendel, 2005, Bluhm et al., 2007). In recent literature, *RAS* genes are increasingly becoming important targets for anti-fungal chemicals due to their direct role in pathogenicity and development (Hast et al., 2011, Perfect, 2017). Due to these developments and the fact that *F. circinatum* is of global and economical importance, results of this study will be useful in designing management strategies that target fungal genes for the development of anti-fungal chemicals to combat pine pitch canker.

CRediT authorship contribution statement

M.M. Phasha: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **M.J. Wingfield:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition. **B.D. Wingfield:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition. **M.P.A. Coetzee:** Conceptualization, Writing - review & editing, Visualization, Supervision. **H. Hallen-Adams:** Conceptualization, Resources, Writing - review & editing. **F. Fru:** Writing - review & editing, Visualization. **B.S. Swalarsk-Parry:** Writing - review & editing, Investigation. **N. Yilmaz:** Writing - review & editing, Investigation. **T.A. Duong:** Writing - review & editing. **E.T. Steenkamp:** Conceptualization, Methodology, Visualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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