



Pathogenicity of *Berkeleyomyces basicola* and *Berkeleyomyces rouxiae* on cotton (*Gossypium hirsutum*) in Australia

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Abstract

Black root rot of cotton (*Gossypium hirsutum*) caused by *Berkeleyomyces rouxiae* is a major seedling disease in New South Wales, Australia. *Berkeleyomyces rouxiae* is morphologically identical to *B. basicola* and both are present in Australian agricultural systems. However, only *B. rouxiae* has been recovered from diseased-cotton to date. In this study, we for the first time evaluate pathogenicity of carrot-*B. basicola* and cotton-*B. rouxiae* directly on two cotton varieties (cv. Sicot 714B3F and Sicot 746B3F) and carrot (*Daucus carota*) seedlings (cv. Topweight), and marketed carrot roots. Both *Berkeleyomyces* species enabled to infect and induce black root rot symptoms on cotton at two inoculum levels i.e. 10^3 and 10^4 spores/mL potting mix. However, the disease severity based on the necrotic percentage were significantly lower ($p=0.0001$) in seedlings inoculated with 10^3 *B. basicola* spores/mL than that with *B. rouxiae*. In co-inoculated assays, co-infection was detected directly on seedlings of both varieties detected with species-specific PCR assays. In this study, Sicot 714B3F appeared more resilient than Sicot 746B3F although both varieties were severely infected with cotton-*B. rouxiae* isolate (disease severity >90%). On carrot, both species failed to incite black rot symptoms on seedlings. However, carrot-*B. basicola* incited more necroses on marketed carrot roots than cotton-*B. rouxiae*. This study provides better understanding of *B. basicola* and *B. rouxiae* pathogenicity on cotton which can affect their prevalence in the field. Therefore, we propose to adapt alternative surveillance strategies to monitor black root rot pathogen of cotton and further explore the host range of the two *Berkeleyomyces* species that would provide valuable information for disease management.

Keywords *Thielaviopsis basicola* · Host specificity · Cross pathogenicity · Surveillance · Pandemic disease

Introduction

In Australia, cotton (*Gossypium hirsutum* L.) is a summer cash-crop, which is planted mainly in regional areas of New South Wales (NSW) and Queensland. Recently, the planting areas have been also expanding to Victoria, Northern Territory and Western Australia. The exported values were up to 5 billion Australian dollars in the 2022/23 cropping season (Dowling 2022). Diseases such as leaf spot (Nguyen and Le 2024a, 2024b), black root rot (Le et al. 2022a; Nehl et al. 2004), Eutypella wilt (Le et al. 2023; Smith et al. 2022),

Fusarium wilt (Kochman 1995; Le et al. 2022b) and Verticillium wilt (Kirkby et al. 2013; Smith 2020) are of the major yield limiting factors to the Australian cotton production.

Black root rot caused by a soilborne fungus, *Berkeleyomyces rouxiae* is a major seedling disease of cotton in NSW, especially in southern areas (Le et al. 2022a; Nehl et al. 2004). The disease was first reported in northern NSW in the 1988/89 cropping season (Allen 1990), but the disease is now widespread across NSW (Le and Nguyen 2024). Black root rot of cotton is characterized by dark brown to black necrotic lesions on below ground parts, these being lower hypocotyl, main and lateral roots. These necroses on the epidermal tissues will subsequently sloughed off when the plants grow (Allen 2001). Therefore, black root rot is presumptively not associated with seedling mortality directly (Nehl et al. 2004). However, black root rot disease result in stunted and less vigorous seedlings, and subsequently, delayed flowering and maturity (Nehl et al. 2004). Yield losses were commonly reported at approximately 10% (Otto

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2020), but replants were occasionally reported in southern valleys of NSW. Also in southern NSW, black root rot level in some fields has continued to intensify to the point that is no longer economic to grow cotton in infested fields (Le and Stiller 2022).

Berkeleyomyces rouxiae is morphologically indistinguishable from its sister species, *Berkeleyomyces basicola*, which was erected from *Thielaviopsis basicola* (Nel et al. 2018). However, they can be separated using DNA sequences of the *internal transcribed spacer* (ITS) and/or *actin*, *minichromosome maintenance complex component 7* (MCM7), *60 S ribosomal protein RPL10* (60 S) and *RNA polymerase II second largest subunit* (RPB2) (Nel et al. 2018). Prior to the description and acceptance of *Berkeleyomyces* as a novel genus, *T. basicola* had a complex taxonomic classification. The fungus was known as *Torula basicola* (Berkeley and Broome 1850), *Helminthosporium fragile* (Sorokin 1876), *Thielavia basicola* (Zopf 1876), *Milowia nivea* (Massee 1884), and more commonly *Chalara elegans* (Nag-Raj and Kendrick 1975; Nel et al. 2018).

Thielaviopsis basicola was able to infect a wide range of plant hosts with approximately 230 plant species recorded (Pereg 2013). However, host specificity and preference were also reported (Pereg 2011, 2013). For example, Australian *T. basicola* isolates recovered from lettuce were pathogenic to lettuce, bean and cucurbits, but they were not pathogenic to capsicum, celery, cotton, eggplant, parsley, radish or tomato (O'Brien and Davis 1994). When tested on cotton seedlings, *T. basicola* isolates originally recovered from cotton, lupin and pansy were more virulent than those recovered from lettuce and carrot (Pereg 2011). Since the erection of *B. basicola* and *B. rouxiae*, Nel et al. (Nel et al. 2019) speculated that host specificity or preference observed in *T. basicola* could have been associated with the two *Berkeleyomyces* species.

Previously in Australia, *T. basicola* also was reported on lettuce, lupin, pansy, soybean and tobacco (Mondal et al. 2004; O'Brien and Davis 1994; Pereg 2011; Simmonds 1966). This indicated that *T. basicola* was relatively prevalent in Australian agricultural systems. Since the erection of *Berkeleyomyces*, only Australian-*T. basicola* isolates from carrot and cotton were re-identified as *B. basicola* and *B. rouxiae*, respectively (Le et al. 2022a; Nel et al. 2018). However, parallel pathogenicity assessments of *B. basicola* and *B. rouxiae* have not been evaluated. In addition, bias isolation of *B. rouxiae* from diseased cotton in the field (Le and Nguyen 2024) was theorised to be associated with different level of virulence of *B. basicola* and *B. rouxiae* in cotton. In this study, we sought to directly compare virulence of *B. basicola* and *B. rouxiae* on carrot and cotton hosts and to assess if these pathogens were able to co-infect cotton

seedlings. Findings will hope to provide better insights into the pathogenicity of *B. basicola* and *B. rouxiae*.

Materials and methods

Fungal cultures and cotton varieties

Four *B. basicola* isolates (Basi 1–4) and one *B. rouxiae* BRR4 were selected for their pathogenicity assays. *B. basicola* isolates were recovered from black rot of carrot and identified using sequences that were retrieved from the MCM7 gene (GenBank accession numbers PV171483 - PV171486). *B. rouxiae* BRR4 was recovered from black root rot cotton and characterised by Le et al. (2022a). All isolates were growing on half-strength PDA (Difco) at 25 °C without light. Spores were collected at four weeks after incubation.

According to Le and Stiller (2022), BRR resistance is not available in Australian commercial cotton varieties. We selected two varieties (cv. Sicut 714B3F and Sicut 746B3F) to assess the pathogenicity of *B. basicola* and *B. rouxiae*. Sicut 714B3F was identified as normal-density seeds, which often resulted in better seedling vigour. On the other hand, Sicut 746B3F was classified as low-density seeds (CSD 2024). To minimise the effect of chemical treatments on pathogenicity of *B. basicola* and *B. rouxiae*, black cotton seeds were used in all assays. The black seeds were provided by Cotton Seed Distributors Ltd.

Assay 1

In this assay, spores of *B. basicola* and *B. rouxiae* collected from four-week-old cultures were directly inoculated into commercially pasteurised potting mix (Searles®). The fungal cultures were flooded with 20 mL of sterile water and gently interrupted with sterile disposable L-shaped spreaders to release the spores. The spore suspension was collected and enumerated using a haemocytometer. The adjusted spore suspension was individually inoculated into the potting mix to obtain a final concentration of 10^4 spores/mL.

Black cotton seeds were sown directly into inoculated potting mix which was contained in 140 mm dia. pots. There were 12 seeds/pot and four replicate pots/treatment, including the negative control without pathogen inoculation. All pots were arranged randomly. Unless otherwise stated, all assays were undertaken in temperature-controlled growth room at 16–25 °C for night and daytime, respectively. The photoperiod was 12 h. All pots were watered daily until the experiment was terminated for disease assessment at six weeks after planting. Upon termination, the cotton seedlings were gently removed from the pots and washed free of potting mix debris. The disease severity based on percentage of

necroses of below ground parts, including lower hypocotyls, and tap and lateral roots was visually scored for individual seedlings (0 for healthy to 100% necroses). Additionally, dry biomass of the seedlings was recorded at two days after drying at 45 °C.

Assay 2

Our unpublished data revealed that the BRR inoculum (kDNA copies/g) did not increase significantly when we recycled the potting mix in the first three consecutive assays. Therefore, in this repeated assay, we recycled the infested potting mix in the assay 1. However, the inoculum level was lowered ten times by mixing one part of the infested potting mix with nine parts of new clean potting mix (Searles®). The assay was carried out in the same manner as described above and terminated for disease and dry biomass assessment at six weeks after planting.

Assay 3

This co-inoculated assay was assessed at two inoculum levels. Spore suspension of *B. basicola* Bas1 and *B. rouxiae* BRR4 was co-inoculated into pasteurised potting mix (Searles®) to obtain a final concentration of 10^4 and 10^3 spores each/mL. The assay was carried out in the same manner as described above and terminated for disease and dry biomass assessment at six weeks after planting.

Assay 4

In this assay, pathogenicity of *B. basicola* Bas1 and *B. rouxiae* BRR4 were assessed on a fast-growing carrot (cv. Topweight) at two inoculum levels i.e. 10^4 and 10^3 spores/mL. The potting mix was thoroughly mixed with spore suspension to achieve desirable inoculum levels. As in the previous assays, carrot seeds were sown directly into the inoculated potting mix, 12 seeds/pot, and four replicate pots per treatment. The carrot was grown until edible roots started to form (nine weeks after planting) before termination for disease scoring and biomass assessment. Carrot roots were separated from the leaves and kept in moistened bags at 23 ± 2 °C for up to four weeks to stimulate the black root rot development on the root surface. Disease severity was scored based on the black necrotic percentage of the roots. Additionally, dry biomass of the seedlings was recorded at three days after drying at 45 °C.

Assay 5

Due to the absence of black rot symptoms on carrot in the assay 4, marketed carrot roots were obtained from a local

supermarket for this dipping inoculation assay. Carrot (25–40 g each) was thoroughly washed under running tap water and surface decontaminated with 25% household bleach (1% sodium hypochlorite) for 5 min. They were then rinsed twice with sterile water and peeled with a peeler before inoculation. The peeled carrot was dipped into spore suspension at 10, 100 and 1000 spores/mL of *B. basicola* Bas1 and *B. rouxiae* BRR4 for 5 min and transferred into moisten zip bags. For each treatment, there were five replicate carrot roots per bag and the experiment was repeated twice. Control treatment was inoculated with sterile water. All experiments were conducted at room temperature (22 ± 2 °C). Number of necroses were enumerated at five days after inoculation.

All recorded disease severity and dry weight were subjected to analysis of variance (ANOVA) and graphing using Graphpad Prism (ver. 9.3.0).

Re-isolation and re-identification

In assay 1 and assay 2, a representative diseased seedling from each replicate pot was subjected to pathogen re-isolation using 5% carrot juice agar (Le 2021). Recovery rate for each *Berkeleyomyces* isolate was recorded. All recovered colonies were transferred onto half-strength PDA and grown in darkness at 25 °C for two weeks. Mycelial and spore masses were collected and subjected to DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega) and molecular identification using specific PCR assays developed by Nakane and Usami (2020). However, due to a mismatch (C/T) in the forward primer bas1MCM7_F, we failed to amplify *B. basicola* using the bas1MCM7_F/bas1MCM7_R primer pair. In this study we used a slightly modified bas1MCM7_FM primer to specifically detect *B. basicola* (Table 1).

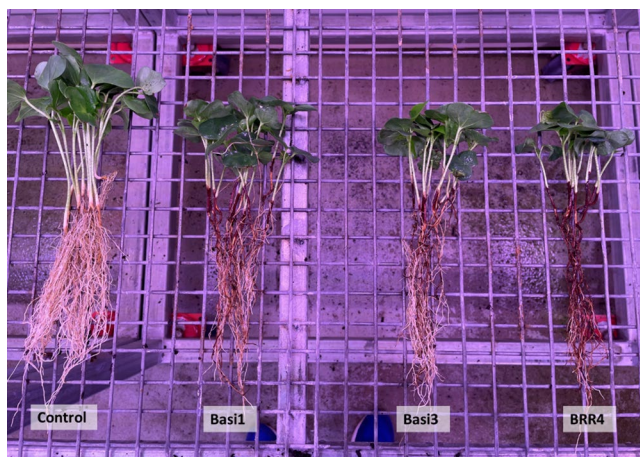
In assay 3, one and five arbitrarily selected seedlings per replicate pot inoculated with 10^4 and 10^3 spores/mL, respectively were subjected to pathogen re-isolation and identification using the above specific PCR assays. Additionally, in an attempt to confirm if co-infection occurred during the course of the assay, a small amount (15–30 mg) of diseased tissue from these selected seedlings was subjected to DNA extraction directly using the Wizard® Genomic DNA Purification Kit. We employed nested PCR to complement the sensitivity of the above specific PCR assays to detect *Berkeleyomyces* directly from diseased-plant DNA extracts. The diseased-plant DNA extracts were first subjected to PCR amplification with the Cer-MCM7F/Cer-MCM7R primer pair (De Beer et al. 2014) (Table 1). The obtained PCR amplicons were used as templates for subsequent specific PCR assays.

In assay 4 and 5, selected carrot roots that developed disease symptoms after incubation in moistened bags were

Table 1 List of primers used in this study to specifically detect *B. basicola* and *B. rouxiae*

Primer name	Primer sequence (5' _3')	Specificity ¹	Annealing temperature (°C)	References
basiMCM7_FM	CGAGTGCAAGAGCACT	<i>B. basicola</i>	50	This study ¹ Nakane and Usami (2020)
basiMCM7_R	TCAGCGAATAGTCCACT			
rouxMCM7_F	CGAGTGCAAGAGTACC	<i>B. rouxiae</i>	50	Nakane and Usami (2020)
rouxMCM7_R	TCAGCGAATAATCCACC			
Cer-MCM7F	ACICGIGTITCIGAYGTNAAGCC	<i>Ceratocystis</i> spp.	55	de Beer et al. (2014)
Cer-MCM7R	TTRGCAACACCAGGRTCACCCAT			

¹ In this study, we slightly modified the primer developed by Nakane and Usami (2020) to correct the mismatched (C/T), which was bolded in the primer sequence

**Fig. 1** Seedlings exhibited different levels of vigour and necroses on below ground hypocotyls, and tap and lateral roots at six weeks after inoculation with 10^4 spores/mL with either *B. basicola* (Basi1 and Basi3) or *B. rouxiae* (BRR4)

subjected to pathogen recovery using the same protocol as described in assay 1 and 2. Then, the recovered pathogens were subjected to the specific PCR for confirmation.

Results

Assay 1

During the six weeks of the experiment, cotton seedlings from control treatment remained healthy looking above ground and no black necroses were observed on below ground hypocotyls, and tap and lateral roots (Fig. 1). On the other hand, vigour of seedlings inoculated with either *B. basicola* or *B. rouxiae* was greatly affected. The seedlings started wilting at four weeks. At six weeks after planting, the seedlings were significantly stunted and seedling death was also noticed. Upon termination for disease severity assessment, characteristic black necroses of black root rot

Table 2 Analyses of variance based on the effects of individual isolates representing two *Berkeleyomyces* species on means black root rot severity on two cotton varieties in assay 1 and assay 2; and the effect of inoculum levels on number of necroses expressed on Carrot roots inoculated with the two *Berkeleyomyces* species in assay 5

Assay 1			
ANOVA for disease severity (%)			
Model effect	Df	F	P
Isolates	4	43.04	0.0001
Varieties	1	75.86	0.0001
Isolates x Varieties	4	5.31	0.0024
Assay 2			
Isolates	4	620.2	0.0001
Varieties	1	1.29	0.2651
Isolates x Varieties	4	1.87	0.1419
Assay 5			
Isolates	1	15.16	0.0004
Inoculum	1	67.13	0.0001
Isolates x Inoculum	1	8.39	0.0064

were present in all inoculated seedlings. Disease severity (%) was significantly different among isolates and between two varieties ($p=0.0001$) (Table 2). On both varieties, disease severity was lowest on seedlings inoculated with *B. basicola* Basi3 and highest on those inoculated with *B. rouxiae* BRR4 (Fig. 2). Both pathogens were recovered and identified accordingly; thus, confirming the pathogenicity. Recovery rate of *B. rouxiae* was 100% in comparison to 32% of *B. basicola* (Table 3).

Dry weight (g/plant) was significantly different between the control and *Berkeleyomyces*-inoculated treatments ($p<0.0001$). In both varieties, *Berkeleyomces* infection reduced the dry weight by half to that of the control. Seedlings from cv. Sicot 714B3F appeared more resilient and had greater dry biomass accumulation than those from cv. Sicot 746B3F (Fig. 3).

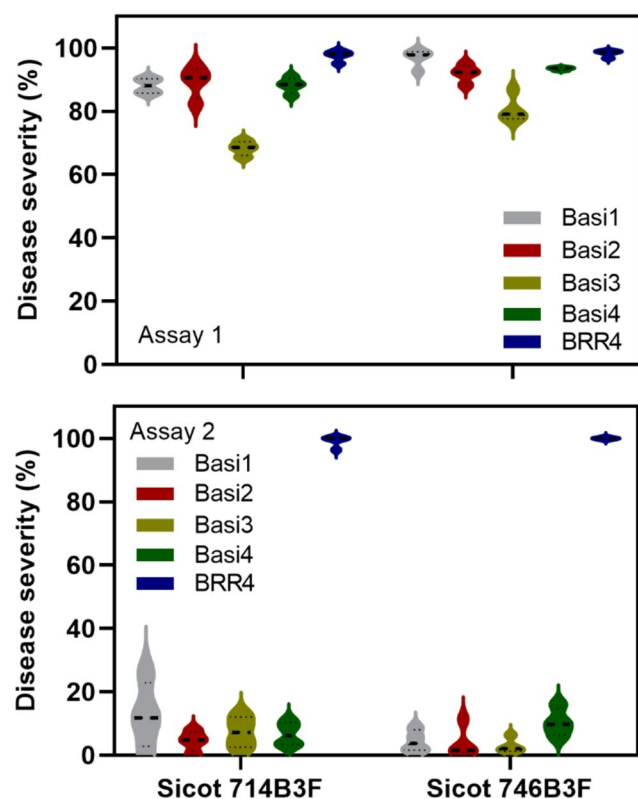


Fig. 2 Disease severity based on percentage of necroses of below ground hypocotyls, tap and lateral roots recorded at six weeks after planting. In assay 1, potting mix was inoculated with 10^4 spores/mL of either *B. basicola* or *B. rouxiae*. In assay 2, infested potting mix from assay 1 was recycled and diluted 10 times with new clean potting mix. Note: due to healthy looking and lack of necroses recorded on the control seedlings (water inoculation), the disease severity data from the control were excluded from statistical analyses

Table 3 Number of *B. basicola* and *B. rouxiae* isolates recovered from diseased tissue in four independent assays

Assays ¹	No. <i>Berkeleyomyces</i> isolates ²	No. of <i>B. basicola</i> ³	No. of <i>B. rouxiae</i> ³
1	23 (55%)	9 (32%)	14 (100%)
2	91 (68%)	53 (58%)	38 (90%)
3	315 (94%)	0	315 (94%)
4	No recovery	No recovery	No recovery
5	12 (100%)	6 (100%)	6 (100%)

¹*B. basicola* and *B. rouxiae* were inoculated individually in assay 1 and 2, and were co-inoculated in assay 3 on cotton. Assay 4 and 5 assessed the virulence of *B. basicola* and *B. rouxiae* carrot seedlings and detached roots, respectively

²Total number of isolates recovered from *Berkeleyomyces*-inoculated treatments. Numbers in the parentheses are the recovery percentage of the pathogen from the diseased tissue

³*B. basicola* and *B. rouxiae* were confirmed using the specific PCR primer sets as listed in Table 1

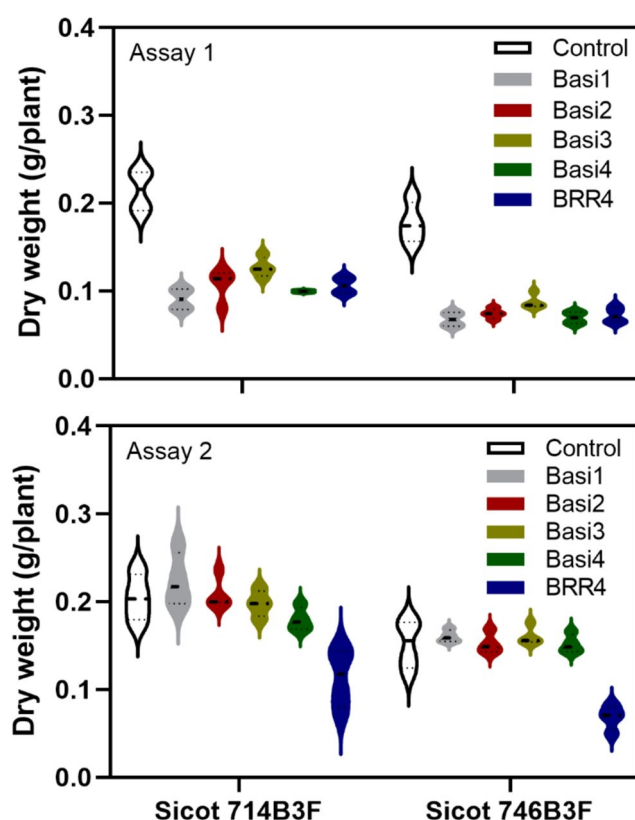


Fig. 3 Dry weight (g/plant) of seedlings recorded at six weeks after planting. In assay 1, potting mix was inoculated with 10^4 spores/mL of either *B. basicola* or *B. rouxiae*. In assay 2, infested potting mix from the preceding assay 1 was recycled and diluted 10 times with new clean potting mix

Assay 2

When lowered the inoculum load by 10 times, seedling health and vigour of the above ground were not visually different among the control and *B. basicola*-inoculated treatments. Wilted, stunted and dead seedlings were only noticed on the *B. rouxiae*-inoculated treatment (Fig. 4). Upon termination for disease assessment, black necroses were observed on all *Berkeleyomyces*-inoculated treatments. On *B. basicola*-inoculated seedlings, light to moderate black necroses were observed mainly on the below ground hypocotyls. However, more profound necroses on all below ground parts were recorded on *B. rouxiae*-inoculated seedlings (Fig. 4). Disease severity was significantly different between *B. basicola* and *B. rouxiae*-inoculated seedlings, but not different among *B. basicola* treatments (Fig. 2). The disease severity recorded on cv. Sicot 714B3F was lowest (4.5%) on *B. basicola* Basi2 and highest (99.1%) on *B. rouxiae* BBR4. Similarly, the highest disease score on cv. Sicot 746B3F was recorded on *B. rouxiae* BBR4-inoculated seedlings. We improved the recovery rate of *B. basicola* by



Fig. 4 Seedlings exhibited different levels of vigour and necroses on below ground hypocotyls, and tap and lateral roots at six weeks growing in infested potting mix. Previously infested potting mix with either *B. basicola* (Basi1) or *B. rouxiae* (BRR4) from assay 1 was diluted 10x with new clean potting mix

Table 4 Disease severity (%) and dry weight (g/plant) recorded at six weeks after planting in infested potting mix co-inoculated with both *B. basicola* and *B. rouxiae* at two inoculum levels

Inoculum	Disease severity (%)		Dry weight (g/plant)	
	Sicot 714B3F	Sicot 746B3F	Sicot 714B3F	Sicot 746B3F
10 ⁴ spores/mL	100 a	100 a	0.06 b	0.03 b
10 ³ spores/mL	97.7 b	99.3 a	0.10 a	0.05 a
P values	0.001	0.160	0.001	0.043

processing the samples in the same day. The recovery rate increased to 58% (Table 3).

Due to low level of disease on *B. basicola*-inoculated seedlings, dry weight was not significantly different between the control and *B. basicola*-inoculated treatment (Fig. 3). In both varieties, the dry weight was lowest on *B. rouxiae*-inoculated treatment and significantly different from others (Fig. 3).

Assay 3

At six weeks after inoculation, all seedlings co-inoculated with both pathogens at 10⁴ spores/mL appeared irreversible wilted and dead. Subsequently, the disease severity was all rated at 100% in both varieties (Table 4). At lower concentration (10³ spores/mL), the co-inoculated seedlings appeared slightly healthier looking. First true leaves started to emerge on some seedlings. However, the mean disease severity was recorded at 97.7% and 99.3% on cv. Sicot 714B3F and Sicot 746B3F, respectively (Table 4). Subsequently, dry weight of seedlings inoculated with 10³ spores/mL was higher than those inoculated with 10⁴ spores/mL (Table 4).

Table 5 Detection of *B. basicola*, *B. rouxiae* and mix infection directly on diseased seedlings using the species-specific nested PCR

Inoculum load ¹	<i>B. basicola</i>	<i>B. rouxiae</i>	Mix infection
10 ⁴ spores/mL	2 (25%)	4 (50%)	2 (25%)
10 ³ spores/mL	4 (10%)	23 (58%)	4 (10%)

¹ At 10⁴ spores/mL, there was a few numbers of seedlings remained, so only eight arbitrarily selected seedlings (1/replicate pot) were subjected to DNA extraction for the direct detection of the pathogen in the diseased seedlings, while at 10³ spores/mL that was up to 5 seedlings per replicate pot (total 40). Numbers in parentheses indicate the frequency of detection

A total of 315 putative *Berkeleyomyces* isolates were recovered from all treatments (two varieties x two inoculum levels). The recovery frequency was 94%. All recovered isolates were identified as *B. rouxiae* using the specific PCR assay (Table 3). However, *B. basicola* was detected directly from diseased seedlings with the frequency of 25% and 10% when inoculated with 10⁴ and 10³ spores/mL, respectively (Table 5). A similar detection rate of co-infection with two pathogens was also observed (Table 5).

Assay 4

At nine weeks after planting in infested potting mix, all carrot plants remained healthy-looking. Upon termination, no obvious black root rot symptoms were visible on edible root surfaces both from the control and inoculated roots. Subsequently, dry biomass weight was not significantly different among treatments (Table S1). At four weeks after incubation in moistened bags at 23±2 °C, no typical black root rot symptoms were observed on the root surfaces. However, the incubation was terminated due to soft rot development. There was no recovery of putative *Berkeleyomyces* isolates (Table 3).

Assay 5

At five days after inoculation, dark gray to black necroses incited by *B. basicola* Basi1 and *B. rouxiae* BRR4 were observed on the inoculated carrot roots, while the control remained clean and healthy looking (Fig. 5). Due to the overgrowth of *B. basicola* Basi1 on the carrot roots inoculated with 1000 spores/mL suspension (Fig. 5), number of necrotic lesions and colonies of *B. basicola* Basi1 were not enumerated and subsequently excluded from ANOVA analyses. There was a significant interaction between isolates and inoculum loads (10 and 100 spores/mL) ($p=0.006$, Table 2). Visually, *B. basicola* Basi1 incited a significantly higher number of lesions on inoculated carrot roots than *B. rouxiae* BRR4 ($p=0.03$ and 0.002 when inoculated with 10 and 100 spores/mL, respectively). For example, mean number of lesions were recorded at 10.5 and 21.4 per carrot root



Fig. 5 Black necroses occurred on carrot root surface at five days after inoculation at room temperature (22 ± 2 °C). Carrot roots were inoculated with carrot-*B. basicola* Basi1 and cotton-*B. rouxiae* BRR4 at

three concentrations (10, 100 and 1000 spores/mL). The control was treated with sterile water only

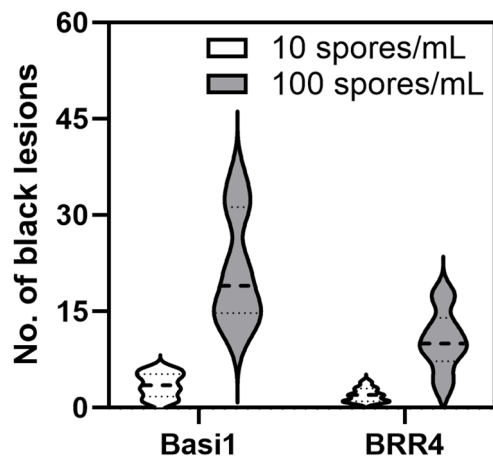


Fig. 6 Number of necrotic lesions recorded on carrot roots at five days after inoculation with *B. basicola* Basi1 and *B. rouxiae* BRR4 at 10 and 100 spores/mL suspension

when inoculated with 100 spores/mL of *B. rouxiae* BRR4 and *B. basicola* Basi1, respectively (Fig. 6). Both pathogens were recovered and confirmed the identity using the species-specific PCR assays (Table 3).

Discussion

In this study, we for the first time directly compared the aggressiveness of *B. basicola* and *B. rouxiae* on cotton and carrot seedlings, and carrot roots to provide a better understanding of their pathogenicity. Both species were pathogenic to cotton; however, *B. rouxiae* was significantly more aggressive, especially at low level of inoculum (10^3 spores/mL). Additionally, both species were able to co-infect

cotton at both inoculation levels. On carrot seedlings, neither species incited visible black root rot symptoms on seedlings and edible roots nine weeks after inoculation. Black rot was generally known as a post-harvest disease of carrot (Cavalcante et al. 2022; Cheah et al. 1996; Weber and Tribe 2004). However, characteristic black rot symptoms were not observed on the edible root surfaces at four weeks post-harvest when incubated in moistened bags. In an additional assay 5, we revealed that *B. basicola* enabled to incite more disease on carrot roots than *B. rouxiae* by dipping the carrot roots into the pathogen spore suspension.

Both *B. basicola* and *B. rouxiae* were described from the well-known *T. basicola*, which can infect an array of host plant species (Pereg 2013). However, host specificity or preference was also reported on *T. basicola* (O'Brien and Davis 1994; Pereg 2011, 2013). Nel et al. (2019) proposed that such observations were probably associated with two different *Berkeleyomyces* species that were originally erected from *T. basicola*. In Australia, only *B. rouxiae* was recovered from diseased-cotton seedlings (Le and Nguyen 2024). Therefore, *B. basicola* originally recovered from carrot were assessed for their pathogenicity in our study. Pereg (2011) previously reported that the Australian cotton-*T. basicola* isolate was more virulent to cotton seedlings than the carrot-*T. basicola* isolate and vice versa. Collective evidence from our assays have indicated that both carrot-*B. basicola* and cotton-*B. rouxiae* exhibited a certain degree of host preference.

The bias recovery of *B. rouxiae* from diseased cotton in the field (Le and Nguyen 2024) was probably either due to an absence of *B. basicola* in cotton soil or a weak infection capability of *B. basicola* to cotton. Therefore, alternative approaches such as baiting or direct soil plating (Allen

1990; Le et al. 2024; Nehl et al. 2004) for *Berkeleyomyces* isolation should be adopted to monitor the prevalence and distribution of these two species in cotton fields. Additionally, environmental DNA metabarcoding method can be employed to rapidly and accurately capture fungal diversity in cotton soil (Kestel et al. 2022) that allows for identification and occurrence of *B. basicola*. Although *B. basicola* may not have a significant biosecurity implication to Australian agricultural systems as a whole, the fungus is not a known cotton pathogen. Therefore, it is important for the Australian cotton industry to remain highly vigilant since we were able to demonstrate the pathogenicity of both *B. basicola* and *B. rouxiae* in artificially inoculated assays. Especially, we also demonstrated the co-infection of both species in cotton seedlings at two inoculum loads (10^3 and 10^4 spores/mL). In the co-inoculation assay, we were able to detect *B. basicola* directly from inoculated seedlings although it was at low frequency and it was co-infected with *B. rouxiae*. However, during the re-isolation, only *B. rouxiae* was recovered. We suspect that the current handling protocol of black root rot of cotton may not be efficient. Therefore, the above alternative approaches should be employed to better monitor black root rot pathogen of cotton.

Nehl et al. (2004) reported that black root rot did not associate directly with cotton seedling mortality in the fields. However, death of cotton seedlings associated solely to severe BRR was reported in cotton fields in Arizona between the 1986 and 1988 seasons (Mauk and Hine 1988). During the experimentation, we constantly observed death of seedlings when inoculated with 10^4 spores/mL of *B. basicola*, while *B. rouxiae* was more virulent and caused seedling death at both inoculum levels. Le and Stiller (2022) noticed some cotton crops in southern valleys in NSW succumbed during establishment stage due to severe black root rot infection. As a result, the crops were terminated and replanted. Cotton-*B. rouxiae* was highly virulent to cotton and resulted in seedling death even at 10^3 spores/mL. It is desirable to have a better understanding of lower inoculum levels on black root rot expression on cotton, which will be helpful for risk assessments and disease predictions.

B. basicola is morphologically indistinguishable from *B. rouxiae* and only is differentiated by DNA sequencing (Nel et al. 2018). In this study, we successfully employed specific primers developed by Nakane and Usami (2020) to identify *B. basicola* and *B. rouxiae* without an aid of DNA sequencing. Additionally, we complemented the sensitivity of the detection assay using nested-PCR to directly detect the two pathogens as well as their co-infection in inoculated seedlings. However, the specific primers resulted in the PCR amplicons at the same size; hence, they were not desirable for simultaneous detection.

According to Le and Stiller (2022), Australian commercial cotton varieties lack gene(s) that mediates resistance against black root rot pathogen. In fact, both tested varieties (cv. Sicot 714B3F and Sicot 746B3F) were severely infected with cotton-*B. rouxiae* isolate at both inoculum levels. However, Sicot 714B3F appeared more resilient and accumulated more biomass than Sicot 746B3F during the course of the experiments. This could be due to nature of seed traits since Sicot 714B3F was known to have better vigour than Sicot 746B3F (CSD 2024). It is known that black root rot impacts cotton seedling vigour (Allen 1990; Mauk and Hine 1988; Nehl et al. 2004); however, the vice versa effect remains little understood. For the future, we recommend that research should be conducted into the influence of seedling vigour on sustaining black root rot infection in cotton.

In conclusion, both carrot-*B. basicola* and cotton-*B. rouxiae* displayed a certain degree of host preference to their original host crops and were pathogenic to two tested cotton varieties at both inoculum levels. However, they failed to incite disease symptoms on carrot seedlings. Both *Berkeleyomyces* species were able to co-infect cotton seedlings, but *B. basicola* was outgrown by *B. rouxiae* during the re-isolation. Therefore, it is warranted to continue to monitor these black root rot pathogens in cotton using alternative approaches such as baiting, direct soil plating, or environmental DNA metabarcoding. Further assessment on host range of these two species is required and will generate important knowledge for disease management.

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Data availability The data that support the findings in this study are contained in the article and supplementary materials.

Declarations

Ethical approval Not applicable.

Conflict of interest The authors declare that they have no competing interests.

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