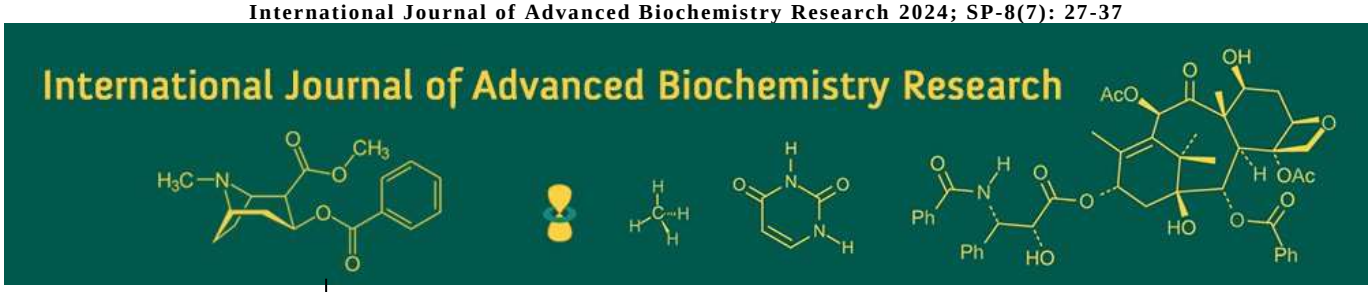

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Studies on the characterization, physiology and transmission of *Botryotinia ricini* (Godfrey) whetzel causes grey mould of castor

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Abstract
Castor is an important non-edible oilseed crop having exceptional oil characteristics for the chemical industry. The crop hosts several diseases, among them grey mold is the most destructive one hindering its productivity. The primary target of the fungus are inflorescence and capsules at any developmental stage. The fungus was isolated from infected castor raceme by standard tissue isolation technique and identified as *Botryotinia ricini* by comparing with the original descriptions. The pathogenicity was established by proving Koch's postulates. PCR amplification with C729 +/- specific primers revealed amplicon of 600bp confirming the molecular diagnosis of *B. ricini*. The physiological studies showed that, pH of 4.0 to 4.5 and temperature around 23 to 25 °C were favourable for the pathogen growth and development in both solid and liquid medium. Seed transmission studies revealed that transmission of the fungus from seed to seedling was facilitated by the cotyledon tip remaining attached to the seed coat after germination, allowing the fungus to inoculate the freshly emerged seedlings.

Keywords: Castor, *Botryotinia ricini,* grey mould, temperature, transmission

Introduction
Castor (*Ricinus communis* L., 2n = 20) is the most important non-edible oilseed crop of arid and semi-arid regions grown across the tropical, sub-tropical and warm temperate regions of the world. It belongs to Euphorbiaceae family, is indigenous to Eastern Africa and originated in Ethiopia which contains 40-60 percent oil content. Castor oil stands unique among the other vegetable oils because of the presence of ricinoleic acid, a hydroxyl fatty acid contributing to high specific gravity and thickness compared to other vegetable oils, with a number of uses (Kaur *et al*., 2020) [1]. Castor oil is rich in triglycerides, mainly ricinolein, which is used predominantly for pharmaceutics, manufacturing of drugs and it possesses exceptional oil features for chemical industries and its global demand is constantly rising at 3-5 percent per annum (Anjani, 2012) [2].

India is the world's largest producer of castor seed and oil, exports shipments of 5.72 lakh metric tons of castor oil (SEA, 2020) [3]. In India, castor cultivation accounts to an area of 9.92 lakh hectares with 19.61 lakh tons of castor oil production (SEA, 2020) [3]. Gujarat, Rajasthan, Karnataka, Orissa and Andhra Pradesh are the prominent castor growing states of India. Gujarat stands first in castor area and oil production in the country accounting for 7.41 lakh hectares with 16.59 lakh tons castor oil production and also leading in terms of productivity with 2231 kg/ha of yield.

Generally, the castor plant is infected by several pathogens such as fungi, bacteria, virus, mycoplasma, and nematode leading to 100 percent yield losses and poor market value of seeds. In India alone, about 80 percent of yield loss in castor crop is attributed to fungal disease (Prasad and Bhuvaneswari, 2014) [4]. Regardless of the tolerance ability of the castor plant to biotic stresses, gray mold, vascular wilt and charcoal rot, are the major diseases that is undermining its productivity (Anjani *et al*., 2004) [5]. Among these, gray mold is the most devastating and difficult disease to manage. It causes direct damage to castor capsules and inflorescence. Under high rainfall and temperature of 25 °C high disease severity was observed. Due to gray mold yield loss could be as high as 100 percent (Anjani, 2012) [6].

Castor gray mold is polycyclic. Hence, infection can reoccur many times when the pathogen is blown by air to a new site in a season.

The *B. ricini* (Godfrey) is the fungal pathogen that causes gray mold in castor and its anamorphic phase is *Amphobotrys ricini* (Prasad & Kumaraswamy, 2017) [7]. The anamorphic form of castor gray mold is capable of causing an epidemic under favourable environmental conditions (Yamuna *et al*., 2015) [8]. In India, gray mold of castor was first reported in Karnataka (Anon, 1921) [9] and appeared as an epidemic in 1985, the pathogen was identified as *Botrytis ricini* (Anon, 1986) [10]. During Kharif 1987, gray mold occurred in an epidemic form causing extensive damage to the crop in erstwhile Andhra Pradesh (Moses and Reddy, 1989) [11] and Tamil Nadu (Anon., 1995) [12], which led to the decline in castor cultivation. Due to gray mold, castor area is reducing in the states of Gujarat, Rajasthan, Andhra Pradesh, Tamil Nadu, Karnataka and Odisha. Disease appears year after year attaining serious proportions limiting castor production in southern states of India (Rao, 1997) [13].

A detailed study on castor gray mold was carried out during 20th century (Godfrey, 1923) [14]. However, the contemporary studies on castor gray mold pathogen for its physiology, management and transmission has to be levitated for its control (Soares, 2012) [15]. Understanding of the disease cycle, pathogen growth requirements in terms of physiological conditions, mode of transmission which helps in developing the suitable ideal management methods which beneficial to castor breeders and growers all around the world.

Materials and Methods

Collection and identification of pathogen

The plants showing typical symptoms of gray mold such as bluish spots on capsules from which yellow liquid oozes out and racemes covered by tan to gray coloured fungal growth, caused by *B.ricini* were collected from fields of K-7 block, ZARS, GKVK, University of Agricultural Sciences, Bengaluru. Gray mold infected capsules were microscopically examined for confirmation of the fungus. Sections of the diseased capsules were made with the help of a sharp blade on a clean glass slide having a drop of lactophenol. The specimen was then covered with a cover slip and observed under compound microscope. After confirmation of the fungus as *Botryotinia*, infected capsules exhibiting typical gray mold symptoms were selected and pathogen was isolated by following standard tissue isolation method.

Isolation and purification of pathogen

The infected capsules were cut into small pieces (5-10 mm long), and were surface sterilized by immersing in 1 percent sodium hypochloride for 2 minutes and then rinsed twice in sterilized distilled water and placed on to the Petri plates containing oat meal agar medium added with streptomycin. The plates were incubated upside down at 23±1 °C temperature for 7 days. The fungal growth emerging from diseased capsule pieces were observed. A loop full of fungal culture developed on oat meal agar medium in Petri plates was taken on a glass slide and observed under microscope for presence of conidia.

Single spore isolation technique was followed for the purification of the pathogen. The spore suspension of the fungal isolate was prepared in sterile distilled water and 1 mL of the suspension from the fungal isolate was spread gently on 15 mL of molten 2 percent water agar. Petri plates were observed for the presence of conidia under compound microscope after 10 days. The spore along with water agar was picked and transferred on oat meal agar medium plates and slants. Petri plates were incubated at room temperature (23±1 °C) and observed for fungal growth and the pure culture so obtained was preserved on oat meal agar medium slants in the refrigerator for further use.

Proving the pathogenicity of the isolated fungus

Pathogenicity test was carried out to establish the isolated fungus capability of producing typical symptoms of gray mold under artificial inoculation condition on castor and also to re-isolate the pathogen to confirm Koch's postulates of pathogenicity. The detached spike of the castor raceme (DCH-519) was placed in a closed polythene humid chamber and these detached spikes were kept in conical flask containing 2 percent sucrose solution. The detached spikes were thoroughly sprayed with sterile distilled water using hand atomizer to ensure free water droplets on the surface of the capsules before inoculation. Inoculum of the pathogen was prepared by using six day old culture of *B. ricini* grown on oat meal agar medium. Conidia of the pathogen were harvested by flooding sporulating cultures with sterile distilled water and gently scraping the surface with a sterile needle. The resultant suspension was filtered through a muslin cloth and the conidial concentration was adjusted to 10^6 conidia mL^{-1} using a haemocytometer. Conidial suspension (10^6 conidia mL^{-1}) was spray inoculated on detached racemes of castor by using an atomizer sprayer.

The inoculated racemes were placed in a growth chamber at 25±1 °C temperature and above 90 percent humidity and observations were made at regular intervals for the symptom development. The symptoms appeared after 5-6 days of inoculation. The organism was re-isolated from these infected detached spikes showing gray mold symptoms and the culture obtained was compared with the original culture for confirmation.

Molecular characterization

The mycelium of both the species collected from the liquid cultures in potato dextrose broth after 7 days of incubation was filtered through Whatmans No-40 filter paper. The mycelia were then dried completely by pressing in between folds of preautoclaved filter papers. The DNA extraction method was standardized and certain steps were optimized to produce good concentration of DNA using plant DNA isolation kit (CTAB method). Mycelium of 0.5 g was taken and ground in a Pestle and Mortar with nine ml of CTAB extraction buffer, mixed gently by inversion. For 60-90 minutes the tubes were incubated at 65 °C, with occasional inversion.

The samples were allowed to cool by keeping the tubes in a trough of water at room temperature. Five ml of chloroform: isoamyl alcohol (24:1) was added, the tubes were gently to mix the content for five minutes. The samples were subjected to spinning in a centrifuge for 15 minutes at 6500 rpm at room temperature. The aqueous layer was transferred to a fresh tube and 25 RNase A (20 mg ml-1) was added. The samples were mixed gently by inversion and incubated for 30 min at room temperature. Six ml isopropanol was added to each tube and mixed gently by inversion until a white fluffy DNA precipitate appeared. The contents were

centrifuged at 6500 rpm for 15 min to pellet the DNA. After 2-3 min, eight ml of cold wash buffer was added and incubated for 20 min at room temperature. The tubes were centrifuged to pellet the DNA at 6500 rpm for 15 min. The supernatant was discarded and eight ml of cold 70 percent ethanol was added to the tube containing the DNA pellet. One ml of elution buffer was added and mixed gently to dissolve the pellet and kept at 4 °C over night. The DNA solution appeared to be turbid after standing overnight at 4 °C and the samples were heated to 65 °C for centrifugation at 6500 rpm for 15 min and the clear supernatant containing DNA was transferred to a fresh 1.5 ml tube discarding the pellet.

Qualitative and quantitative verification of DNA

The quality and quantity of DNA was analyzed by running 2 µl of each sample mixed with 2 µl of 10x loading dye in 1% agarose gel. The DNA was quantified by comparing with the 1 kb size marker (Genei Pvt. Ltd. Bengaluru). The gel was observed under UV light and documented using gel documentation unit.

Polymerase chain reaction (PCR) using C729+/- primer sequence for the Bengaluru culture isolate of *B. ricini*

Molecular identification of fungal species relies on the amplification of the C729+/- specific region (C729+:5'-AGCTCGAGAGAGATCTCTGA-3'C729-:5' CTGCAATGTTCTGCGTGGAA -3') (Rigotti *et al*., 2002) [16] of the fungal genome. Primer for amplification was custom synthesized at Indus Biosolutions, Bengaluru and supplied as lyophilized products of desalted oligonucleotides. PCR programme for selected gene amplification was set in Eppendorf thermal cycler.

Identification of amplified region using AGE

Agarose gel (1.2%) was prepared by dissolving 1.2 g agarose in 100 mL TAE buffer. After cooling, 4 µL EtBr (Ethidium Bromide) was added. Gel was casted in casting tray and wells were made by inserting comb in casting tray. PCR samples together with tracking dye (6X Methanol blue) were loaded in the well. Gel was kept in the electrophoretic unit for 45 minutes at 100W. After 45 minutes, gel was taken out carefully from gel casting tray and placed into InGenius3 gel Doc-Syngene. Gel pictures were taken using Gene sys software and saved in jpg format.

Physiological studies of *B. ricini*

The effect of hydrogen ion concentration (pH) on mycelial growth of *B. ricini* in solid and liquid medium infecting castor. This study was conducted to know the influence of pH on the mycelial growth of *B. ricini* at six pH levels. pH of PDA and PDB was adjusted to *viz.*, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 with 1N Hydrochloric acid (HCl) and 1N Sodium hydroxide (NaOH) by using digital pH meter. Each treatment was replicated thrice. The pH adjusted media was autoclaved at 121.6 °C and 15 lbs pressure for 15 minutes. Fifteen mL of PDA and 50 mL of PDB was poured into Petri plate and conical flask, respectively and was seeded with 5 mm mycelial disc from actively growing cultures and incubated for 7 days at 23±1 °C. Mycelial growth was observed periodically and the diameter of the colony was measured after 10 days of incubation in case of solid media. Observations on growth and sporulation were recorded. The mycelial mat on PDB was harvested and filtered by using previously weighed Whatman No.1 filter paper and air dried at 60 °C in hot air oven until the dry weight readings became constant. The difference in weight of mycelia was averaged and analysed statistically. Effect of temperature on mycelial growth of *B. ricini* in solid and liquid medium infecting castor

The growth of *B. ricini* was tested at eight different temperature levels *viz.*, 15, 20, 22, 23, 25, 28, 30 and 35ºC to know the influence of temperature on mycelial growth of *B. ricini* on both PDA and PDB medium. Fifteen mL of PDA and 50 mL of PDB was poured into Petri plate and conical flask respectively which was seeded with 5 mm mycelial disc from actively growing cultures and incubated for 7 days in the incubator adjusted to required temperature levels.

Each treatment was replicated thrice. After incubation, the mycelial growth and sporulation from solid media were recorded. However, the mycelial mat on PDB was harvested and filtered by using previously weighed Whatman No.1 filter paper and air dried at 60 °C in hot air oven until the dry weight readings become constant. The difference in weight of mycelia was averaged and analyzed statistically.

Transmission of *B. ricini* from seed to seedling in castor

Transmission of *B. ricini* from castor (DCH-519) seeds to seedlings was studied in laboratory as well as in glass house conditions using both naturally infected as well as artificially inoculated seeds.

Paper towel method

For transmission studies under laboratory conditions, 25 each of naturally infected and artificially inoculated castor seeds (sterilized and unsterilized) were placed on paper towel, incubated at 25±2 °C and examined for symptoms expression on seedlings after 10 days. Percent germination, seed rot, seedling mortality and seedling infection were recorded.

Glass house method

The transmission studies under glass house conditions, 5 each of both naturally and artificially infected (sterilized and unsterilized) castor seeds were grown in sterilized soil in plastic pots (20cm×13cm×18cm) and were monitored for seedling growth and symptom expression at 30 days. Percent germination, seed rot, seedling mortality and seedling infection were recorded.

Results

Isolation and identification of *B. ricini* from capsules of castor

Isolations made from collected infected capsules of DCH-519 castor showing typical symptoms of gray mold revealed the presence of *B. ricini*. Colonies of the fungus on oat meal agar medium were irregular, fluffy, radial or in concentric rings, initially hyaline to light brown in colour which later turned dark gray.

The conidiophores were dichotomously branched, cylindrical, straight and pale brown in colour on which conidia were formed. Macroconidia were globose, unicellular and pale brown in colour whereas, microconidia were globose and hyaline. Small black coloured sclerotia were formed on 12th day after inoculation along the edges of the Petri dish. The pure culture of the fungus was obtained

and purified by single spore isolation method on oat meal agar medium (Fig. 1).

Morphological identification of gray mold pathogen of castor

The fungus on oat meal agar medium produced septate and branched mycelium, dichotomously branched conidiophores bearing single celled globose, hyaline to light brown coloured conidia (Fig. 1).

Pathogenicity test

The pathogenicity test was conducted in a closed polythene humid chamber as described in material and methods. The detached spikes of susceptible castor variety, DCH-519, were kept in conical flasks containing 2 percent sucrose solution and inoculated with spore suspension (10^6 conidia mL^{-1}). Initial symptoms were observed on 3rd day of inoculation. The first symptoms of the disease were visible as bluish spots on the capsules. These spots were enlarged covering the entire castor raceme with grayish moldy growth within six days after inoculation (Fig. 2).

Molecular characterization of *B. ricini* infecting castor

Genomic DNA of the fungus *B. ricini* was isolated by CTAB method. The obtained DNA was observed through electrophoresis in 1.2 percent agarose gel and quantification of DNA was done using Nano drop spectrophotometer According to morphological characteristics isolate belonged to *B. ricini* (Hennebert, 1973; Godfrey 1919). For molecular confirmation of the isolate C729+/- specific primer designed specifically for *Botrytis* detection by Rigotti *et al*., 2002 [14] was used.

The DNA of the pathogen causing gray mold was amplified in PCR using C729 forward and C729 reverse specific primers. The amplified product was checked by electrophoresis in 1.2 percent agarose gel. A single band of 600 bp that is specific to *B. ricini* was amplified by the isolate. No band was amplified in the negative control (Fig. 3). The result of this study confirmed the molecular diagnosis of *B. ricini* as the cause of gray mold of castor.

Physiological studies of *B. ricini*

Effect of hydrogen ion concentration (pH) on mycelial growth of *B. ricini* in solid and liquid medium infecting castor

To check the most favourable pH, six different pH levels (pH 4.0-6.5) were used in the study with detailed procedure as mentioned. Both solid (PDA) and liquid media (PDB) were used to study the effect of pH on the pathogen growth (Table 1, Fig. 4A & 4B and Fig 5).

Based on observation, maximum mycelial growth was recorded in pH 4.5 on both solid and liquid medium viz., 86.96 mm and 1.32 mg/100 mL, respectively followed by pH at 4.0 on solid medium (85.29 mm) and in liquid medium. The dry weight of mycelial growth was maximum (1.29 mg /100 mL) at pH 4.0 and 5.0, which are on par with each other and significantly superior over all other pH.

In solid medium the mycelial growth in pH 5.0 and 5.5 was 80.63 and 73.53 mm respectively. The other pH viz., 6.0 and 6.5 also showed good enough mycelial growth. Petri dishes adjusted to pH 5.5 shows highest sporulation and at pH 5.0, 6.0 and 6.5 revealed scanty to moderate sporulation. In liquid medium pH 5.5 shows moderate mycelial growth of 1.21 mg /100 mL and minimum growth was recorded in pH 6.0 and 6.5 as evidenced by 0.79 and 0.39 mg /100 mL respectively (Table 1). The study conducted to understand the suitable pH for the pathogen growth proved that pH between 4.0 to 4.5 was ideal for the pathogen growth and pH 5.5 recorded the highest sporulation.

Effect of temperature on mycelial growth of *B. ricini* in solid and liquid medium infecting castor

The temperature plays an important role in the occurrence and degree of disease severity of gray mold pathogen. In order to know the influence of temperature on growth rate of *B. ricini*, temperature range of 15 to 35 °C were chosen in the study. Here, both solid (PDA) and liquid medium (PDB) were used to study the effect of temperature on the pathogen growth (Table 2, Fig. 4C & 4D and Fig 5B).

In solid medium the mycelial growth of the isolate at 15 °C was 42.16 mm and with the increase in temperature there was also enhancement in the growth of mycelium until 25 °C viz., 78.58 mm at 20 °C, 88.21 mm at 22 °C, reaching maximum growth at 23 °C (89.93 mm), 89.92 mm at 25 °C and at temperature of 28 °C it recorded mycelial growth of 79.34 mm. Mycelial growth of isolate decreased to 77.82 mm at 30 °C and minimum mycelium growth was recorded at 35 °C (39.43 mm). Sporulation observation revealed that there was no sporulation at temperature of 15 and 35 °C but scanty sporulation were observed at 30 °C. Profuse sporulations were recorded at temperature range of 25 to 28 °C. Moderate to good sporulation was recorded at temperature range of 22 to 23 °C. In liquid medium, dry weight of mycelial growth of the isolate increased with increase in temperature from 15 °C (78.58 mm) and reaches maximum at temperature of 23 °C (1.36 mg /100 mL). Under 30 °C dry mycelial weight decreased to 0.65 mg /100 mL and minimal dry weight was observed at temperature of 35 °C (0.20 mg /100 mL).

In the present study, it was observed that with the increase in temperature (up to 25 °C), the fungus showed enhanced growth both in solid and liquid medium. In solid medium profuse sporulation was recorded at temperature range of 25 to 28 °C, there was no sporulation at temperature 15 and 35 °C. The study proves that temperature around 23 to 25 °C are favourable to *B. ricini* growth and disease development.

Transmission of *B. ricini* from seed to seedling

Paper towel method

Seed transmission studies of the pathogen from infected castor (DCH-519) seed to seedling under laboratory conditions in paper towel revealed that, naturally and artificially inoculated seeds with *B. ricini* showed seed rot, abnormal seedlings and in case of severely infected seeds, seedling mortality was observed thus resulting in reduced seed germination. (Table 3, Fig. 6A & 6B).

The germination in naturally infected castor seeds was 11.60 and 6.60 percent in sterilized and unsterilized seeds respectively. In naturally infected seeds, 69.33 and 83.13 percent seed rotting, 14.50 and 25.50 percent diseased seedlings, 14.80 and 25 percent seedling mortality were recorded in surface sterilized and unsterilized seeds respectively. The germination in artificially inoculated castor seeds was 86.60 and 73.30 percent in surface sterilized and unsterilized seeds, respectively. 2.83 and 27.17 percent seed rotting, 6.97 and 9.50 percent seedling infection and mortality was observed in unsterilized seeds.

Whereas, there was no seedling infection and seedling mortality was observed in surface sterilized seeds.

When seed rotting was compared to germination, seedling mortality, and seedling infection, a significant increase in seed rotting was seen in naturally infected seeds. The seeds became hollow and germination dropped dramatically as a result of a severe *B. ricini* infection. Germination percentage was much greater in artificially inoculated seeds compared to seed rotting because the pathogen could not reach the seed owing to the strong seed coat and thus could not affect the seed germination.

Glass house studies

Seed transmission studies of the pathogen from infected castor (DCH-519) seed to seedling under glasshouse conditions in pot culture with soil, revealed similar symptoms that were observed in paper towel method (Table 4, Fig. 6C & 6D).

Germination in naturally infected castor was 20 percent in surface sterilized seeds and there was no germination in unsterilized seeds. 20 and 90 percent seed rotting in surface sterilized and unsterilized seeds respectively, 32.60 percent seedling mortality and 33.30 percent diseased seedlings was recorded in surface sterilized seeds. In unsterilized naturally infected seeds 2.50 percent seedling infection and 12 percent seedling mortality was observed.

In artificially inoculated seeds germination was 86.60 percent in sterilized seeds and 80 percent in unsterilized seeds. Whereas, 2.50 percent seed rot and there was no seedling mortality and seedling infection was recorded was recorded in sterilized artificially inoculated seeds. In unsterilized artificially inoculated seeds, 22 percent seed rotting, 2.50 percent seedling infection and 8.30 percent seedling mortality was recoded. The seed to seedling transmission of *B. ricini* was compared in the two experiments, i.e., paper towel and glass house methods and it was concluded that in naturally infected seeds, seed rotting was significantly higher than seed germination because the seed is ill- filled as the pathogen infected the seed during the early stages of seed formation or at the time of seed maturation and making it rot or hollow.

Discussion

Castor is an important non-edible oilseed crop which contains 40-60 percent of oil, rich in triglycerides, mainly ricinolein. India ranks first in area and production of castor in the world. Diseases are the major biotic stresses for the successful production of castor. Among these, gray mold caused by *B. ricini* a necrotrophic pathogen is a most destructive pathogen which has been reported to cause potential seed yield loss up to 100 percent as it infects the inflorescence directly. Experiments were conducted for the study of gray mold of castor which includes Isolation and molecular characterization of pathogen, physiological studies of pathogen and transmission studies of *Botryotinia* conducted at AICRP (Sunflower) pathology section, UAS, GKVK, Bengaluru.

The first symptom appeared as minute bluish spots on flowers, developing fruits, panicle, leaves and stem from which yellowish drops of liquid exude oozes out. On fruits the symptoms appeared as circular or elliptic, sunken, dark coloured spots which result in rupture of the capsule. The inflorescence or 'spike' is covered with a moldy growth which is gray at first and later turn olive gray, fungal mass become darker as inflorescence becomes older and infection spreads to entire racemes resulting in total destruction of affected tissues. These descriptions are similar to the symptoms described by Godfrey, 1923; Araujo *et al.*, 2007; Basha *et al.*, 2021; Yamuna *et al.*, 2021 [15 -19]. The isolated pure culture of the fungus on oat meal agar medium were stored in oat meal agar medium slants (Sung-Kee *et al.*, 2001) [20].

The morphological characteristics of fungus grown on oatmeal agar displayed septate, branched mycelium with dichotomously branched conidiophores that produced single-celled, globose, hyaline to light brown conidia. These characteristics match the descriptions by Godfrey, 1919; Godfrey, 1923; Hennebert, 1973; Zada *et al.*, 2016; Arutselvan *et al.*, 2020 [21, 15, 22, 23, 24], observed small bluish spots on panicles, leaves and stems of castor infected with castor gray mold. The growth of gray to olive gray mycelium on infected racemes were described as a spider's web.

The result of this study confirmed the molecular diagnosis of *B. ricini* as the cause of gray mold of castor with the primer (C729 forward and C729 reverse specific primers). amplification and its product at 600 bp that is specific to *B. ricini* isolate. The above results was supported by the findings of Rigotti *et al.*, 2002, Khazaeli *et al.*, 2010 and Sarita Kumari *et al.*, 2014 [14, 25, 26] where they used C729+/- specific primer pair for molecular confirmation of gray mold pathogen.

The study conducted to understand the suitable pH for the pathogen growth proved that pH between 4.0 to 4.5 was ideal for the pathogen growth and pH 5.5 recorded the highest sporulation. The study also revealed that the pathogen growth decreased with further increase in pH level. These results are in accordance with Ahlem *et al.*, 2012 [27] who found that pH range from 4 to 5 showed the positive effect on that growth of *B. cinerea* from gray mold of strawberry. Ahmed *et al.*, 2014 [28], wherein they recorded pH favourable for mycelial growth of *B. cinerea* in five isolates of chickpea. Thus, it was concluded that the growth rate of *B. ricini* was favoured between pH ranges of 4.0 to 4.5. Similar result also have been found with the result of Tripathi and Suyal, 2015 [29] on favourable effect of pH range from 4.0 to 5.5 on the growth of *B. cinerea.*

This study observed that the fungus grew better as the temperature increased to 25 °C in both solid and liquid mediums. Profuse sporulation occurred at 25 to 28 °C, but there was no sporulation at 15 and 35 °C. This shows that temperatures around 23 to 25 °C are ideal for *B. ricini* growth and disease development. The results are in accordance with Yasmeen *et al.*, 2003 and Sussel, 2008 [30, 31] wherein they confirmed that temperatures around 25 to 28 °C were favourable for disease development and minimum growth was observed at 35 °C and growth was very slow with poor sporulation with increased temperature, Also Fernandez *et al.*, 2014 [32] reported the maximum growth of all strains of *B. cinerea* at 28 °C. The Fedele *et al.*, 2020 [33] also studies the effect of temperature at 25 °C for maximum growth of *B. cinerea* isolates from grapes.

The seed to seedling transmission of *B. ricini* was compared in the two experiments, *i.e.*, paper towel and glass house methods and it was concluded that in naturally infected seeds, seed rotting was significantly higher than seed germination because the seed is ill- filled as the pathogen infected the seed during the early stages of seed formation

or at the time of seed maturation and making it rot or hollow.

Transmission of the fungus from seed to seedling may be facilitated by the cotyledon tip remaining attached to the seed coat after germination, allowing the fungus to inoculate the freshly emerged seedling Tichelaar, 1967 [34] discovered similar results while working with transfer from seed to seedling of *B. alli* (undifferentiated from *B. aclada*). He showed that the fungus can penetrate the tip of the cotyledon from the seed coat microscopically. Similarly, according to Brewster, 1994 [35] fungal transmission from seed to seedling is aided by the cotyledon tip remaining attached to the seed coat via a haustorium during germination and emergence, when the haustorium collects nutrients from the endodermis. These findings are consistent with those of Koike *et al*., 2006 and Sowley *et al.*, 2010 [36-37] who found that the pathogen spreads from the seed coat while it is still linked to the cotyledon. Similar results has been found with Harold *et al*., 1997; Sowley *et al*., 2010; Singh *et al*., 2011 and Sowley, 2016 [38-40] on different crops infected with *B. cinerae*. As a result, seed surface sterilization reduces seedling infection both in infected and artificially inoculated seeds.

Table 1: Effect of hydrogen ion concentration (pH) on mycelial growth of *B. ricini* in solid and liquid medium infecting castor

Tr. No.	Ph	Mycelial growth (mm)**	Dry weight of mycelia (mg /100 mL)*	Sporulation
T_1	4.0	85.29	1.29	-
T_2	4.5	86.96	1.32	-
T_3	5.0	80.63	1.29	+
T_4	5.5	73.53	1.21	+++
T_5	6.0	69.83	0.79	++
T_6	6.5	50.47	0.39	+
	C.V. (%)	0.83	1.99	
	S.Em ±	0.36	0.01	
	C. D. (P=0.01)	1.11	0.04	

Note: *Mean of three replication in liquid media, **Mean of three replication in solid media -: absent; +: 1-4 scanty; ++: 5-8 moderate; +++: 9-15 good; ++++: >15 abundant. Figures in parenthesis are arcsine transformed values.

Table 2: Effect of temperature on mycelial growth of *Botryotinia ricini* in solid and liquid medium infecting castor

Tr. No.	Temperature	Mycelial growth (mm)**	Dry weight of mycelia (mg /100 mL)*	Sporulation
T_1	15	42.16	0.40	-
T_2	20	78.58	0.50	+
T_3	22	88.21	1.28	++
T_4	23	89.92	1.35	+++
T_5	25	88.87	1.29	++++
T_6	28	89.93	1.36	++++
T_7	30	77.82	0.65	+
T_8	35	39.43	0.20	-
	C.V. (%)	0.61	2.54	
	S.Em ±	0.26	0.01	
	C.D. (P=0.01)	0.79	0.04	

Note: *Mean of three replication in liquid media; **Mean of three replication in solid media-: absent; +: 1-4 scanty; ++: 5-8 moderate; +++: 9-15 good; ++++: >15 abundant.
Figures in parenthesis are arcsine transformed values.

Table 3: Transmission of *Botryotinia ricini* from seed to seedling in paper towel method

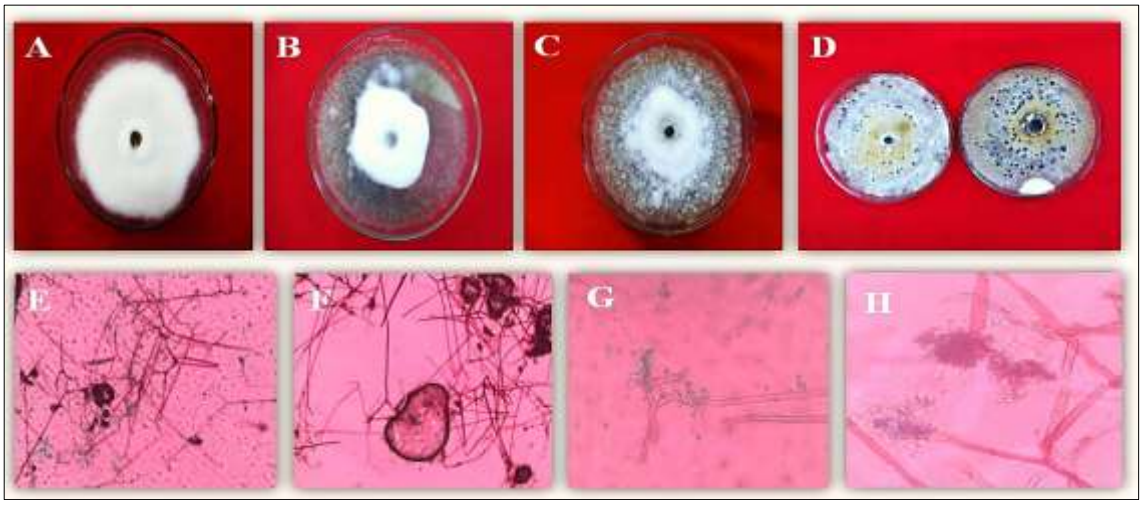
Test parameters	Naturally infected seeds		Artificially inoculated seeds	
	Surface sterilized Seeds	Unsterilized seeds	Surface sterilized seeds	Unsterilized seeds
Germination (%)	11.60 (19.91)	6.60 (14.96)	86.60 (68.53)	73.30 (58.89)
Seed rot (%)	69.33 (56.37)	83.13 (65.75)	2.83 (9.68)	27.17 (31.42)
Seedling infection (%)	14.50 (22.38)	25.50 (30.33)	0.00 (0.00)	6.97 (15.31)
Seedling mortality (%)	14.80 (22.63)	25.00 (30.00)	0.00 (0.00)	9.50 (17.95)
CV (%)	2.07	1.11	3.57	3.06
S.Em ±	0.53	0.29	0.24	0.22
CD (P =0.05)	1.77	0.97	0.80	0.71

Note: Figures in parenthesis are arcsine transformed values.

Table 4: Transmission of *Botryotinia ricini* from seed to seedling in glass house method

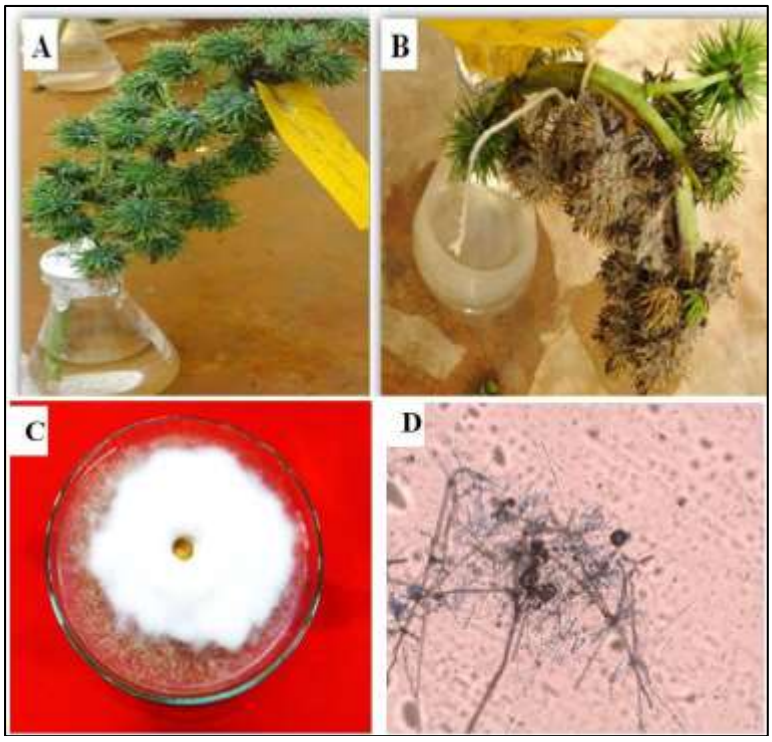
Test parameters	Naturally infected seeds		Artificially inoculated seeds	
	Sterilized seeds	Unsterilized seeds	Sterilized seeds	Unsterilized seeds
Germination (%)	20.00 (26.57)	0.00 (0.00)	86.60 (68.53)	80.00 (63.44)
Seed rot (%)	20.00 (26.57)	90.00 (71.57)	2.50 (9.10)	22.00 (27.97)
Seedling infection (%)	33.30 (35.26)	2.50 (9.10)	0.00 (0.00)	2.50 (9.10)
Seedling mortality (%)	32.60 (34.86)	12.00 (20.27)	0.00 (0.00)	8.30 (16.78)
C.V. (%)	3.64	5.41	6.78	5.48
S.Em ±	0.98	1.05	0.38	0.42
CD (P =0.05)	3.25	3.48	1.24	1.39

Note: Figures in parenthesis are arcsine transformed values.



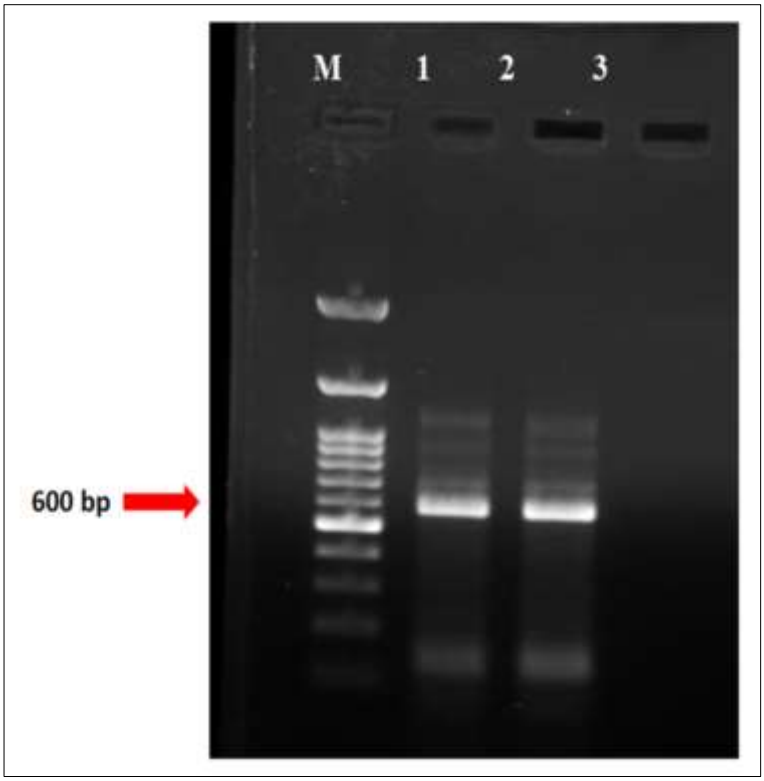
A. Colonies were fluffy and formed in concentric rings; B. Sporulation of *B. ricini*; C. Gray coloured colony; D. Small black coloured sclerotia; E. Microscopic view of mycelium and conidiophore; F. Microscopic view of sclerotia; G. Dichotomously branched conidiophore with septation; H. Macro and microconidia

Fig 1: Cultural and micro morphological characteristics of *Botryotinia ricini*



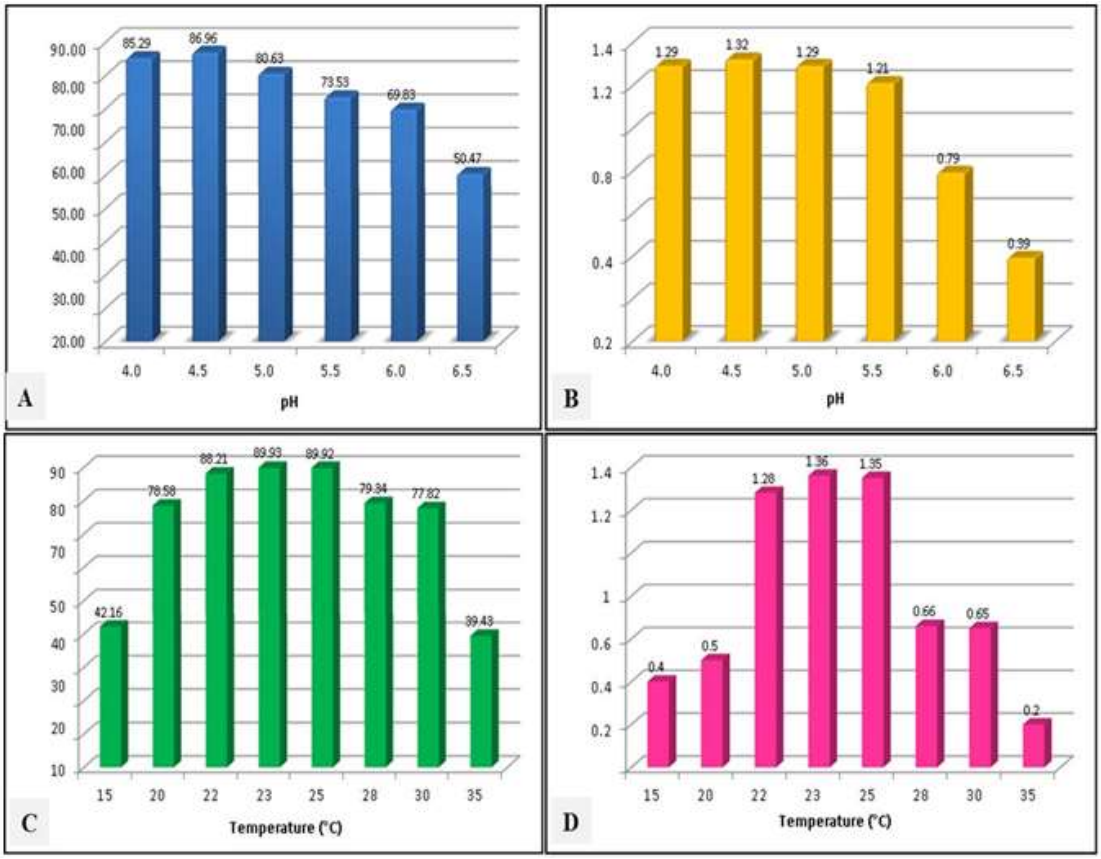
A. Healthy raceme, Inoculated with conidia of *B. ricini*, Re isolated culture, Re isolated conidia with dichotomously branched conidiophore

Fig 2: Pathogenicity of *Botryotinia ricini* on detached castor spikes



Ladder: M. 1Kb; 1 and 2. *B. ricini*; 3. Negative control

Fig 3: Specific region amplication of *Botryotinia ricini* by C729 ± primers

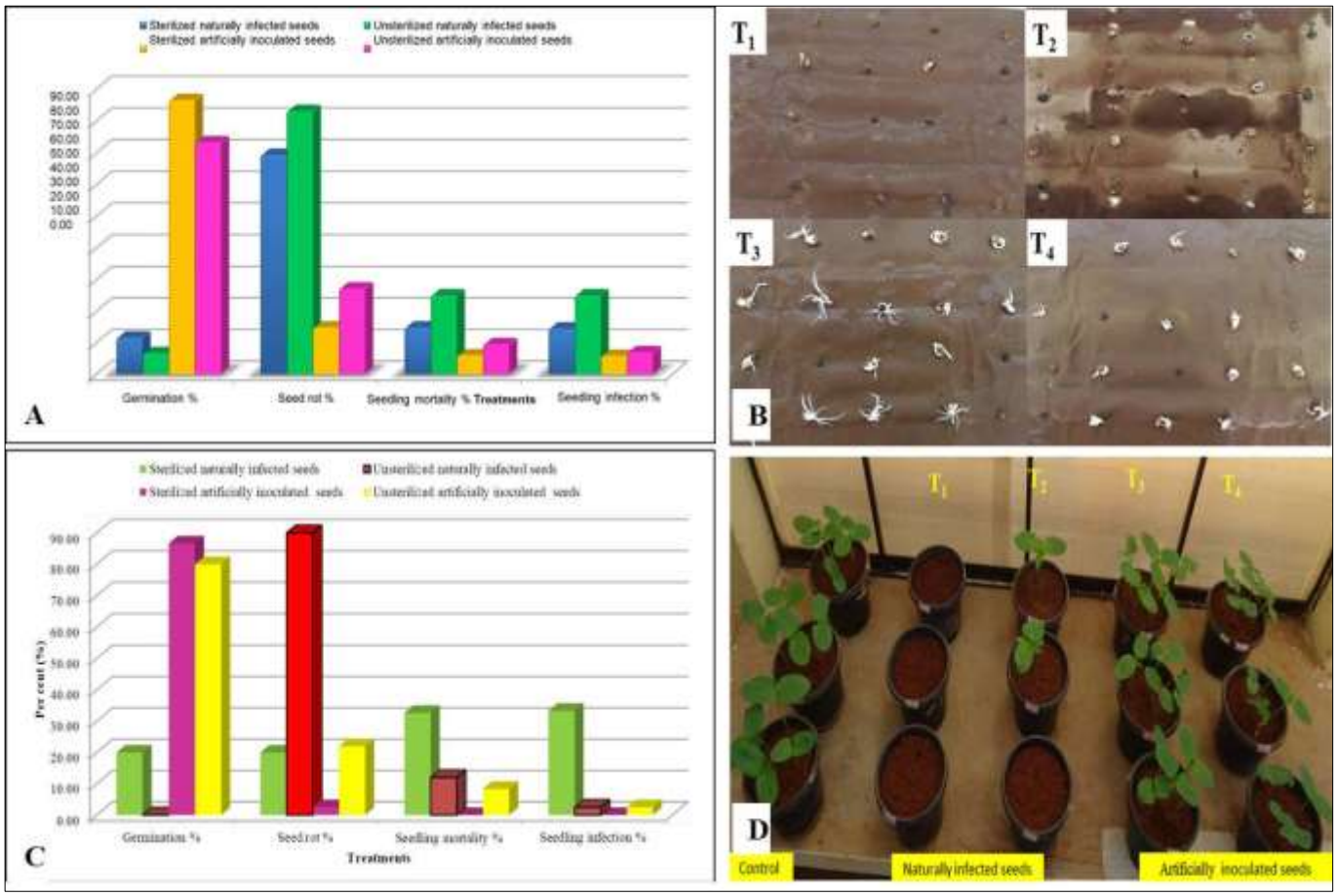


A. Effect of hydrogen ion concentration (pH) on mycelial growth of *B. ricini* in solid medium infecting castor, B: Effect of hydrogen ion concentration (pH) on dry mycelial weight of *B. ricini* in liquid medium infecting castor; C: Effect of temperature on mycelial growth of *B. ricini* in solid medium infecting castor; D: Effect of temperature on dry mycelial weight of *B. ricini* in liquid medium infecting castor

Fig 4: Effect of hydrogen ion concentration (pH) on mycelial growth of *B. ricini in* different media

A: Effect of hydrogen ion concentration (pH) on growth of *B. ricini* on solid and liquid medium infecting castor, **B:** Effect of temperature on growth of *B. ricini* on solid and liquid medium infecting castor

Fig 5: Effect of hydrogen ion concentration (pH) on growth of *B. ricini* on different media



A: Transmission of *B. ricini* from seed to seedling in paper towel method B: Transmission of B. ricini from seed to seedling in paper towel method C: Transmission of *B. ricini* from seed to seedling in glass house method D: Transmission of *B. ricini* from seed to seedling in glass house method (T1 - Unsterilized naturally infected seeds, T2 - Sterilized naturally infected seed, T3 - Unsterilized artificially inoculated seeds, T4- Sterilized artificially inoculated seeds).

Fig 6: Transmission studies of *B. ricini* from seed to seedling through different methods

Conclusion

Studies on castor gray mold caused by *B. ricini* (Godfrey) Whetzel, a well- known necrotrophic pathogen, considered as a most destructive and known to cause potential seed yield loss up to 100 percent was undertaken in the present work, The study proves that temperature around 23 to 25 °C were favorable to *B. ricini* growth and disease development and the pH 4.0 to 4.5 was found to be ideal on both solid and liquid medium. However, maximum mycelial growth was recorded at pH 4.5 (86.96 mm and 1.32 mg/100 mL) and highest sporulation was observed at pH 5.5. PCR amplification with C729 +/- specific primers revealed that a single band of 600 bp that is specific to *B. ricini* was amplified in the isolate which confirmed the molecular diagnosis of *B. ricini* as the cause of gray mold of castor. The transmission of the fungus from seed to seedling facilitated by the cotyledon tip remaining attached to the seed coat after germination, allowing the fungus to inoculate the freshly emerged seedlings. These studies have been helped in Understanding of the disease cycle, pathogen growth requirements, identification of resistant cultivars appropriate, optimal management techniques used, will be beneficial to castor breeders and growers all around the world.

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Conflict of interest

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

Ethical statement

All the experimental procedures involving only on plant species were conducted following the University of Agricultural Science, Bangalore institutional guidelines. There are no human and animal subjects/trials conducted in this article and informed consent is not applicable.

Disclosure statement

The authors declare that there are no financial/commercial conflicts of interest.

Author contributions

C. P. Manjula (Conceptualization [supporting], Data curation [lead], Formal analysis [lead], Investigation [lead], Visualization [lead], Writing -original draft [lead]), B. Prathibha (Supervision [supporting], Validation [equal], Writing -review & editing [equal]), Yamanura. (Supervision [supporting], Validation [supporting]), Divyashree (conceptualization [supporting], Supervision [lead], Formal analysis [lead], Visualization [lead]) K. B. Palanna (Conceptualization, Data curation, Writing -review & editing- supporting) and G. Punith (Writing-original draft [supporting], Data curation [supporting]).

References

1. Kaur R, Biswas B, Kumar J, Jha MK, Bhaskar T. Catalytic hydrothermal liquefaction of castor residue to bio-oil: effect of alkali catalysts and optimization study. Ind Crops Prod. 2020;149:112359.
2. Anjani K. Castor genetic resources: a primary gene pool for exploitation. Ind Crops Prod. 2012;35(1):1-14.
3. Sea. Sea castor crop survey 2019-20. The Solvent Extractors Association of India, Mumbai, India; c2020. p. 4-12.
4. Prasad RD, Bhuvaneswari R. A modified medium for improved sporulation of gray mold pathogen, Botryotinia ricini (Godfrey) Whetzel in castor (*Ricinus communis* L.). J Oilseeds Res. 2014;31(1):79-81.
5. Anjani K, Raoof MA, Ashoka Vardhana Reddy V, Hanumanata Rao C. Sources of resistance to major castor (*Ricinus communis* L.) diseases. Plant Genet Resour Newsl. 2004;137:46-48.
6. Anjani K. Castor genetic resources: a primary gene pool for exploitation. Ind Crops Prod. 2012;35(1):1-14.
7. Prasad RD, Kumaraswamy B. Simple technique for screening of gray mold disease in castor. Int J Pure Appl Biosci. 2017;5(4):1653-1656.
8. Yamuna C, Kishore Varma P, Prasad RD, Vijaya Lakshmi NDK. Morphological and molecular characterization of anamorph associated with gray mold of castor (*Ricinus communis* L.). J Oilseeds Res. 2015;32(1):63-67.
9. Anonymous. Inflorescence and stem rot. Bangalore, Karnataka. Proceedings of the second meeting of mycological workers in India held at Pusa on 20th Feb 1921 and following days. Supdt. Govt. Printing, Calcutta; c1921.
10. Anonymous. Annual report castor, 1985-86, Directorate of Oilseeds Research, Rajendranagar, Hyderabad, India; c1986.
11. Moses GJ, Reddy RR. Gray rot of castor in Andhra Pradesh. J Res Andhra Pradesh Agric Univ. 1989;17:74-745.
12. Anonymous. Annual progress report of castor, All India Coordinated Research Projects on Oilseeds. Directorate of Oilseeds Research, Rajendranagar, Hyderabad. 1995.
13. Rao HC. Castor area declines in south coastal Andhra Pradesh. DOR Newsl. 1997;3:3.
14. Godfrey GH. Gray mold of castor bean. J Agric Res. 1923;23(9):679-715.
15. Soares DJ. Gray mold of castor: a review. In: Cumagan CJR, editor. Plant Pathology; c2012. p. 219-240.
16. Rigotti S, Viret O, Gindro K. Two new primers highly specific for the detection of Botrytis cinerea Pers. Fr. Phytopathol Mediterr. 2002;45(3):253-260.
17. Araujo EA, Suassuna DN, Coutinho MW. Doenças e seu manejo. In: O Agronegócio da Mamona no Brasil. Azevedo DMP, Beltrao NEM, editors. Embrapa Informacao Tecnologica, Brazil; c2007. p. 283-303.
18. Basha SA, Ramya V, Begum AS, Raghavendra G, Ramanjaneyulu AV, Kumar MV. Evaluation of Pseudomonas fluorescens strains, fungicides and non-conventional chemicals against Botryotinia ricini causing gray mold disease in castor. Int J Bio-Res Stress Manag. 2021;12(4):339-347.
19. Yamuna C, Varma PK, Prasad RD. Management of castor gray mold using Trichoderma species. J Pharmacogn Phytochem. 2021;10(1):111-115.

20. Sung-kee H, Wan-gyu K, Weon-dae C, Hong-gi K. Occurrence of gray mold in castor bean caused by Botrytis cinerea and *Amphobotrys ricini* in Korea. Plant Pathol J. 2001;17(6):357-360.
21. Godfrey GH. Sclerotinia ricini parasitic on castor bean (*Ricinus communis* L.). Phytopathology. 1919;9:565-567.
22. Hennebert GL. Botryotinia and botrytis-like genera. Persoonia. 1973;7(2):183-204.
23. Zada N, Irshad G, Naz F, Gulzar A, Shahid M, Aslam MF, Ghuffar S. Morphological and cultural characterization of Botrytis cinerea causing gray mold disease of lentil crop from Pakistan. Pak J Phytopathol. 2016;28(2):249-254.
24. Arutselvan R, Devi GU, Prasad RD, Sarada C. Effect of temperature and culture media on mycelial growth and sporulation of Botryotinia ricini causing gray mold of castor; c2020.
25. Khazaeli P, Zamanizadeh H, Morid Bayat H. Morphological and molecular identification of Botrytis cinerea, causal agent of gray mold in rose greenhouses in central regions of Iran. Int J Agric Sci Res. 2010;1(1):19-24.
26. Sarita Kumari, Tayal P, Sharma E, Kapoor R. Analyses of genetic and pathogenic variability among Botrytis cinerea isolates. Microbiol Res. 2014;169:862-872.
27. Ahlem H, Mohammed E, Badoc A, Ahmed I. Effect of pH, temperature and water activity on the inhibition of Botrytis cinerea by Bacillus amyloliquefaciens isolates. Afr J Biotechnol. 2012;11(9):2210-2217.
28. Ahmed AU, Zaman S, Mazid MA, Rahman MM, Sarkar MMR, Arbia L, Ud-deen MM, Kabir G. Studies of Botrytis cinerea causing Botrytis gray mold disease in chickpea (*Cicer arietinum* L.). J Bio-Sci. 2014;22:69-76.
29. Tripathi HS, Suyal U. Botrytis gray mold of chickpea (*Cicer arietinum* L.). Recent Adv Diagn Manag Plant Dis. 2015;69-79.
30. Yasmeen M, Raoof MA, Rana Kausar. Host range of Botryotinia ricini Godfrey, castor gray mold pathogen. In: National Symposium on Plant Pathogens Diversity in Relation to Plant Health. Hyderabad, India: Osmania University; c2003
31. Sussel AAB. Epidemiologia do mofo-cinzento (*Amphobotrys ricini* Buchw.) da mamoneira. PhD thesis. Universidade Federal de Lavras, Lavras, Brazil. 2008;109.
32. Fernández JG, Fernández Baldo MA, Sansone G, Calvente V, Benuzzi DA, Salinas E, Raba J, Sanz Ferramola MI. Effect of temperature on the morphological characteristics of Botrytis cinerea and its correlation with the genetic variability; c2014.
33. Fedele G, Brischetto C, Rossi V. Biocontrol of Botrytis cinerea on grape berries as influenced by temperature and humidity. Front Plant Sci. 2020;11:559208.
34. Tichelaar GM. Studies on the biology of Botrytis alli on Allium cepa. Neth J Plant Pathol. 1967;73:157-60.
35. Brewster JL. Onions and other vegetable Alliums. Wallingford, Oxon, UK: CAB International; 1994.
36. Koike ST, Gladders P, Paulus AO. Vegetable diseases: a color handbook. Plant Protection Handbook Series; c2006. p. 59-61.
37. Sowley EN, Dewey FM, Shaw MW. Persistent, symptomless, systemic, and seed-borne infection of lettuce by Botrytis cinerea. Eur J Plant Pathol. 2010;126(1):61-71.
38. Harold JFS, Fitt BD, Landau S. Temperature and effects of seed-borne Botrytis cinerea or Alternaria linicola on emergence of linseed (*Linum usitatissimum*) seedlings. J Phytopathol. 1997;145(2-3):89-97.
39. Singh S, Kaur I, Sirari A. Role of infected seed and soil in disease transmission of Botrytis grey mould in chickpea. J Food Legumes. 2011;24(4):335-37.
40. Sowley EN. Detection of seed to seed transmission of Botrytis cinerea in lettuce. Pak J Phytopathol. 2016;28(2):115-120.