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A SIMPLE METHOD FOR SCREENING GRAY MOLD OF CASTOR (*Ricinus communis* L.) UNDER ARTIFICIAL CONDITIONS

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ABSTRACT

***Botryotinia ricini* (Godfrey) Whetzel, the gray mold pathogen of castor, inflicts serious crop losses in short periods. The present study was aimed at to develop a season-independent screening technique for gray mold in castor. Conidia from *in vitro*-raised pure culture of the anamorph *Amphobotrys ricini* were sprayed on separated mature as well as immature capsules of 4 castor genotypes (DCS 9, RG 2787, RG 3309 and RG 3216 R), placed in glass petriplates and incubated at 22-24ºC for 7 days. Genotype DCS-9 was highly susceptible to the disease, while genotype RG 3216 R exhibited least susceptibility. Immature capsules were more susceptible to disease than mature capsules. RNA degradation occurred in treated samples 2 days after treatment and control gene (UBQ) expression was unaffected till 4 hr post-inoculation. Thus a reproducible, lab-based, season-independent, screening technique using petri-plates and severed castor capsules was developed for differential gene expression analysis of candidate genes involved in early infection and elucidating host-pathogen interactions in castor gray mold.**

Key words: *Amphobotrys ricini*, *Botryotinia ricini*, castor, gene expression, gray mold disease, sporulation, susceptible

INTRODUCTION

Castor (*Ricinus communis* L.) is a non-edible oilseed crop of arid and semi-arid regions and a potential source of biofuel (Sreenivas *et al*., 2011). Pests and diseases limit the productivity of castor and cause heavy yield losses (Anjani *et al*., 2004; Rao *et al*., 2012). One of the major destructive diseases of castor is gray mold caused by *Botryotinia ricini* (Godfrey) Whetzel, a necrotrophic fungus which infects seed-bearing spikes directly, sporulates and turns the whole spike into a spore mass, devastating the crop within 2-3 days under favourable conditions (Dange *et al*., 2005; Soares, 2012). In India, gray mold incidence is season-dependent, mostly occurs in October-November when there is continuous precipitation for 2-3 days with temperature <28°C and causes serious economic losses in castor growing belts of Andhra Pradesh, Telangana and Tamil Nadu (Soares, 2012). The fungus produces characteristic dark plane-convex elongated sclerotia, survives in soil as sclerotial mass during off-season and attacks castor spikes when favourable conditions return. The anamorphic state (asexual reproductive phase) of *Botryotinia ricini* named as *Botrytis ricini* N.F. Buchw (Buchwald, 1949) and later as *Amphobotrys ricini* (N.F. Buchw) Hennebert (Hennebert, 1973) is responsible for disease epidemics. At present, neither effective control measures nor completely resistant cultivars to castor gray mold are available.

The mechanism of infection is unknown in *Amphobotyrs ricini*, the gray mold pathogen of castor, but is known in a related species *Botrytis cinerea* (Laleve *et al*., 2014). Growth and penetration of *Botrytis* sp. in host tissues depends on inoculum size, water and nutrient availability, cuticle features, presence of exudates on floral organs, abundance of natural openings as well as size and age of wounds (Holz *et al*., 2007). The fungus penetrates cuticle probably using both mechanical and chemical processes either directly using germ tube or after enzymatic lysis of cuticle (Orellana and Thomas, 1962). *A. ricini* sporulates in spikes of castor only during favourable environmental conditions and hence any *in vivo* study on disease and screening for resistance to pathogen is a season-dependent process. Understanding the molecular mechanisms underlying host–pathogen interactions may provide some information on infection strategy of gray mold pathogen in castor and thereby help in devising disease-management strategies. Though the fungus sporulates and produces profuse conidia under natural conditions in field, sporulation under *in vitro* conditions in normal culture media is sparse, due to profuse mycelial growth and sclerotia formation in culture plates. Hence, culture method and culture medium was optimized for *in vitro* sporulation of *Amphobotrys ricini* (Parvathy *et al*., 2013). The present study was aimed to develop a lab-based, season-independent screening method for gray mold infection in castor under artificial conditions using *in vitro* raised *A. ricini* spores and separated capsules of castor, which could be used for differential gene expression analysis or host-pathogen interactions in castor gray mold.

MATERIALS AND METHODS

Plant material

Two susceptible genotypes of castor *viz*., variety DCS 9 and germplasm accession RG 2787 and two partial resistant germplasm accessions RG 3309 and RG 3216 Red (red capsule type, hereafter referred to as RG 3216 R), showing partial resistance/decreased infection based on field screening at ICAR-IIOR) [Anjani *et al*., 2004], were chosen for the present study. The plants of 4 genotypes were raised and maintained at ICAR-IIOR, Rajendranagar farm during 2012-13, at plant spacing of 60 x 60 cm and with 10 plants per row.

Culture media and conditions

Pure culture of *Amphobotrys ricini* was isolated from infected capsules of castor genotype DCS 9 at ICAR-IIOR, Rajendranagar (India) in 2012 and conidial suspension maintained as glycerol stock (Parvathy *et al*., 2013). The culture was revived in BRS (*Botryotinia ricini* sporulation) culture medium comprising of 20 g V8 juice broth powder, 5 g yeast extract, 10 g peptone, 20 mM potassium phosphate buffer (pH 7.0), 10% (w/v) capsule extract and 15 g agar per litre [pH 5.5]. The BRS medium was autoclaved at 15 psi for 20 min. and streptomycin 50 mg L^{-1} added after autoclaving, to avoid bacterial contamination. Spore suspension [50 µL] from glycerol stock was added to 50 µL sterile water in the centre of petri-plates and swirled for even spread, inside a laminar air flow under sterile conditions. The plates were sealed with parafilm®, kept in a tray with moistened blotting sheets, covered with aluminum foil and incubated in a BOD incubator (Caltan® Seed Germinator) at 22±1°C, with 75% humidity. The blotting sheets in tray were moistened every 24 hr till sporulation.

Preparation of A. ricini culture for infection

Fresh spores were harvested from plates in 1 mL sterile distilled water in Eppendorf tube. The spore clumps were broken in an ultrasonic bath sonicator (S.V. Scientific) at 15 min. intervals for 1 hr to form a fine suspension. The spores were counted by hemocytometer (Superior, Germany) under a compound microscope (Nikon H600L, Japan). Final dilution was made with distilled water to a final concentration of 10^6 spores mL^{-1} and Tween 20 added @ 0.2 mL L^{-1} in spray solution and used for spraying the capsules.

Screening of castor lines for resistance to A. ricini in culture plates

Pathogenicity of isolate was determined by spraying *A. ricini* culture on cut spikes of highly susceptible genotype DCS 9, prior to screening. Whole spikes were cut from both susceptible (DCS 9 and RG 2787) and resistant/partially resistant genotypes (RG 3309 and RG 3216 R) from plants raised at IIOR farm at Rajendranagar in 2012. Mature and immature spikes of each genotype were collected separately. Immature spikes had all the capsules in capsule setting stage [1.0 to 1.5 weeks after flower opening (depending on the line/genotype)] and soft to touch. Mature spikes had majority of fully formed capsules with well-developed seeds and was hard to touch. The spikes were thoroughly washed in running water and capsules separated. The sterilized glass petri-plates (19 cm dia.) were wiped with 70% ethanol and 2 circular blotting sheets of same size placed on the bottom and moistened. The plate was marked into two equal regions along diameter for immature and mature capsules. Ten each of mature and immature capsules of each genotype were placed in each half of the petri-plate separately. Control and pathogen-infected capsule treatments were separately maintained. The capsules were adequately moistened with distilled water. The control plates were sprayed with distilled water while the treated set was sprayed with *A. ricini* spore suspension. The plates covered with aluminum foil were incubated at 22±1°C for 7 days. To maintain high moisture level in plates, distilled water was sprayed daily. Disease progression was monitored at regular intervals and photographs taken using Canon digital camera. The disease progression in terms of per cent infection was calculated as percentage of number of capsules infected out of total capsules treated on different days after treatment. The experiment was conducted in a completely randomized design and each treatment replicated thrice. Effect of pricking was tested by comparing infection in spikes taken in glass petri-plates by 7 days, with and without pricking, before spraying *A. ricini* spores.

Screening based on cut spikes and whole plants under glass house conditions

For cut spike experiments, mature and immature spikes of DCS 9, RG 2787, RG 3309 and RG 3216 R were cut from field-grown plants and placed in 250 mL conical flasks containing water. For whole plant screening, whole plants of 4 genotypes were raised in a net house. Staggered planting of RG 3309 and RG 2787 was done for simultaneous screening of infection on spikes. At least 5 pots per genotype were transferred to glasshouse and spikes on whole plant sprayed with *A. ricini* as above, along with cut spikes and disease progression observed.

Isolation of RNA

Mature and immature capsules (3 per sample) from control and treated (infected) plates of susceptible genotype DCS 9 were collected in liquid nitrogen at regular intervals, from another set of screening experiments, after recording observations. The samples were stored at -80°C till use. RNA was extracted from the capsules using TRIzol® method as per manufacturer's instructions. The quality and quantity of RNA was estimated by agarose gel electrophoresis in 1X TAE and Nanodrop® Spectrometer, respectively. RNA degradation in infected samples was determined from the intactness of rRNA bands after loading equal quantity of RNA from samples at different time intervals in 1% agarose gel.

Reverse transcription-PCR (RT-PCR)

The primers of 2 control genes (along with 23 candidate genes involved in infection process) were manually designed using sequences downloaded from NCBI, based on homology with other organisms. RNA samples were treated with DNase I as per manufacture's (Invitrogen) instructions by adding DNase I enzyme: 1 µL 10X DNase buffer: 1 µL RNA: 0.5-1.0 µg (volume in µL adjusted for equal concentration of RNA in all samples) and the total volume made upto 10 µL with diethyl pyro carbonate (DEPC) water. The reaction mixtures were kept at 37 ºC for 15 min. DNase I was inactivated by adding 1 µL EDTA and incubated for 2 min. at 75ºC.

One-step RT-PCR was carried out using Primescript® one step RT-PCR kit as per the manufacturer's (Takara) instructions to verify the expression of control gene *ubiquitin* of castor (*UBQ*) using forward primers FP UBQ: 5′ GCGGAAAGATGCAGA TCTTCG3′ and reverse primer RP UBQ: 5′ TCCTCTCAATCGCAGCACCAG 3′ in collected samples. The RT-PCR reaction was made with 12.5 µL 2X 1 step RT-PCR buffer, 1 µL prime script® enzyme, 1.5 µL forward and reverse primers (10 µM) to final concentration of 0.6 µM, 2 µL RNA (DNAse treated) and the total volume made upto 25 µL using DEPC-treated water or RNase-free water. The PCR reactions were carried out in a thermocycler (Eppendorf®) using equal amount of DNase treated RNA from all samples at cycling conditions of reverse transcription for cDNA synthesis at 50ºC for 30 min., initial denaturation at 94ºC for 2 min., followed by 30 cycles of denaturation at 94ºC for 30 sec., primer annealing at 55ºC for 45 sec., extension at 72ºC for 1 min. and final extension at 72ºC for 10 min. Equal volume (12 µL) PCR products were loaded in each lane along with 100 bp ladder (Genei, India) in 1% agarose gel in 1X TAE at 85 V. The gel was documented in gel doc (Syngene, G box, UK).

RESULTS AND DISCUSSION

Screening in culture plate under artificial conditions

The pathogenicity of *A. ricini* was tested before screening experiments. Symptoms of infection in severed capsules initiated as browning of spines followed by browning of capsule epicarp, tissue softening and sporulation releasing brownish ooze, which were similar to that in whole plants. Immature capsules of susceptible genotype DCS 9 treated with *A. ricini* spores, exhibited tissue browning and brownish exudation by 3rd day, whereas genotypes RG 2787 and RG 3309 showed browning by 4th day. All immature capsules of susceptible genotypes DCS 9 and RG 2787 were infected and showed profuse sporulation by 7th day (Fig. 1A). Symptoms of infection or sporulation of *A. ricini* were not observed in control plates by 7 days. However, in RG 3309, only 75% immature capsules showed infection, while no infection was observed in immature capsules of RG 3216 R. Treated mature capsules in susceptible genotypes DCS 9 and RG 2787 turned black by 4th day after spray; whereas it started by 3rd day in case of RG 3309 and RG 3216 R. All mature capsules of genotypes DCS 9 and RG 2787 were completely black with spores noticed at the tip of long spines. About 60-70% mature capsules of RG 3309 exhibited complete blackening of epicarp, while in RG 3216 R, all mature capsules turned black without sporulation. Thus immature capsules were more susceptible and prone to infection than mature capsules, in susceptible varieties. Reduced wall integrity facilitates pathogen success (Cantu *et al.*, 2008) and may be the reason for more susceptibility in immature capsules. As such, immature capsules alone may be used for screening for gray mold infection, while mature capsules of same genotype could be used as treated control. Comparison of treated capsules of 4 castor genotypes with untreated control revealed prominent blackening of mature capsules in treated samples than in control. Genotype RG 3216 R exhibited resistance to infection and had black mature capsules, in control. The results of lab-screening in culture plates were compared with cut spike (Fig. 1B) and whole plant screening conducted in glass house (Fig. 1C) simultaneously. Similar to culture plate technique, infection appeared by 4th day in susceptible varieties and sporulation was complete by 7 days, causing maximum damage to spikes. RG 3216 R was resistant. Unlike the cut spike and whole plant screening in glass house, where there are more chances of cross-infection of conidia from infected capsules to untreated susceptible varieties, in petri-plate screening, proper untreated controls can be maintained for molecular biology experiments.

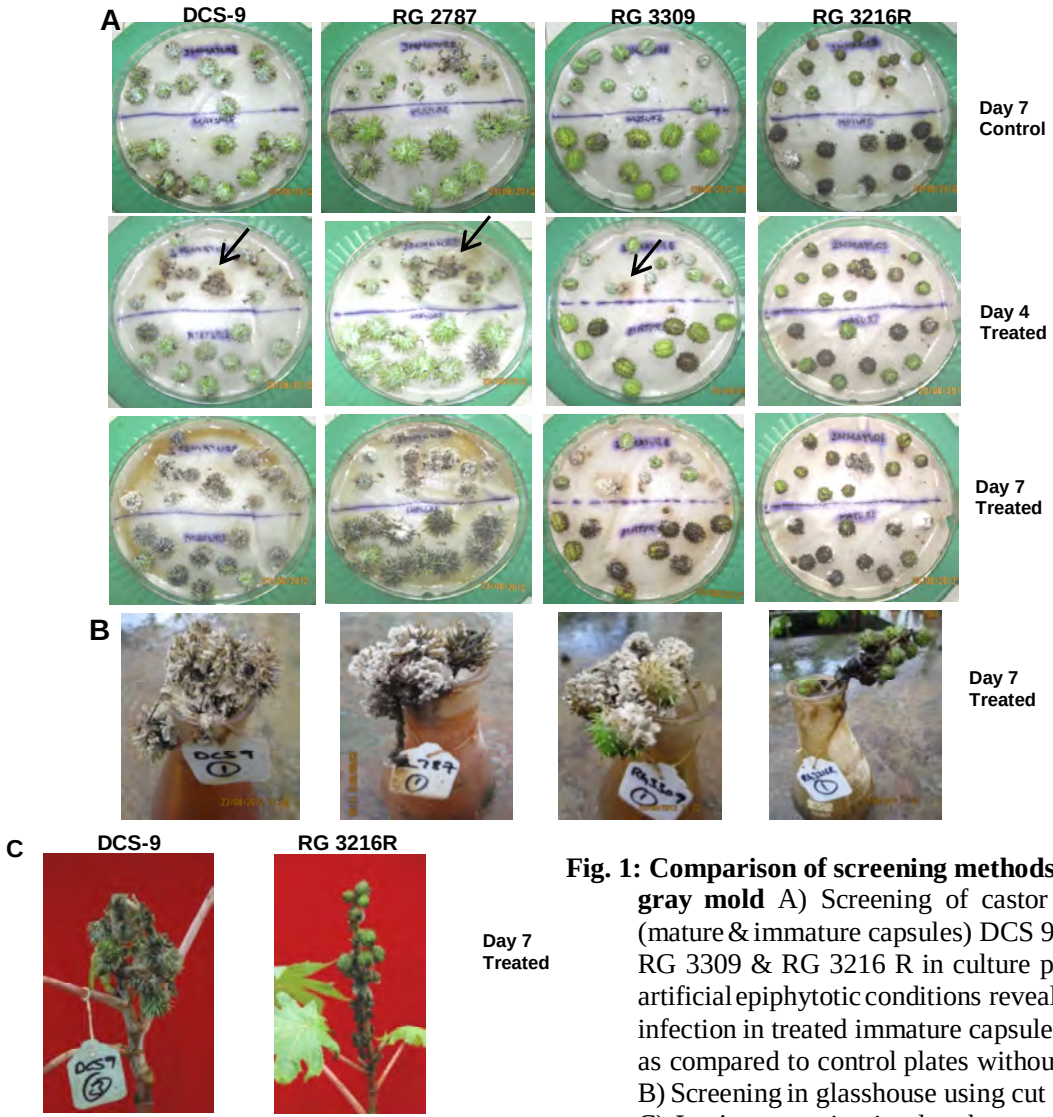

Fig. 1: Comparison of screening methods for castor gray mold A) Screening of castor genotypes (mature & immature capsules) DCS 9, RG 2787, RG 3309 & RG 3216 R in culture plates under artificial epiphytotic conditions reveals complete infection in treated immature capsules by 7 days as compared to control plates without infection; B) Screening in glasshouse using cut spikes; and C) *In vivo* screening in glass house using whole plants.

The disease progression of gray mold in culture plate screening, depicted as infection percentage on different days after treatment, showed that RG 2787 picked up infection slowly, but by 7th day the whole plant was completely infected. Genotype DCS 9 is highly susceptible followed by RG 2787 and RG 3309 (Fig. 2). Thus, a simple, reliable, lab-based (*in vitro*) season-independent screening technique was developed for screening castor genotypes or varieties for gray mold susceptibility/ resistance under artificial conditions.

A petri-plate technique for testing the pathogenicity and inducing fungal sporulation, not achieved by conventional media, was reported by Christiensen (1988), where fungi isolated from the diseased seedlings of forage Brassicas were inoculated onto the agar surface of each petri plate, close to the developing seedlings. A diagrammatic scale was developed to assess gray mold infection caused by *A. ricini* in castor bean, by incubating 5 mm mycelia discs on disinfected castor clusters/spikes, packed in moistened foam trays in climatic chambers at 25°C and 80% relative humidity (Chagas *et al*., 2010). Wounded sites of sterilized apple fruits harvested at red ripe stage were inoculated with 10 µL water suspension containing conidia of *Botrytis cinerea* and incubated at high humidity for 3 days for disease development in artificial conditions (Blanco-Ulate *et al*.,

2014). In castor, however considering the small size of capsules, hardness of epicarp of capsules and presence of spines on the surface, conidial suspension spray without any wounding is better. The whole spikes darkened and 2-3 capsules exhibited sporulation in 6 days in both treatments viz., with and without pricking or wounding (data not shown). *Amphobotrys ricini* does not depend on wounds for entering host cells or tissue during infection process as pricking does not obviously result in increased infection. The gray mold pathogen used other means to enter the tissue and probably uses enzymatic lysis (Lionetti *et al*., 2007; Kikot *et al*., 2008) or oxidative processes for infection similar to other necrotrophic *Botrytis* sp. (Govrin and Levine, 2000; Shetty *et al*., 2008). Under lab conditions, where the spores are concentrated in a small area with many capsules, with highly conducive condition for infection, effect of surfactant Tween 20 may be insignificant, while surfactant is a must for field screening. Presence or absence of spines in capsules may not be a mechanism of resistance since RG 3309 with short sparse spines was also infected. The components in epicarp may be a factor of resistance. When used for RNA extraction or gene expression studies, sterile conditions have to be maintained during all operations, such as opening the plates in laminar airflow and use of sterile distilled water for preparation of spore suspension or for spraying inside plates. Surface sterilization of capsules has to be carried out with sodium hypochlorite 2% for 30 sec. to ensure infection of *A. ricini* alone and to avoid changes in expression profile due to other parasitic/saprophytic organisms or opportunistic pathogens.

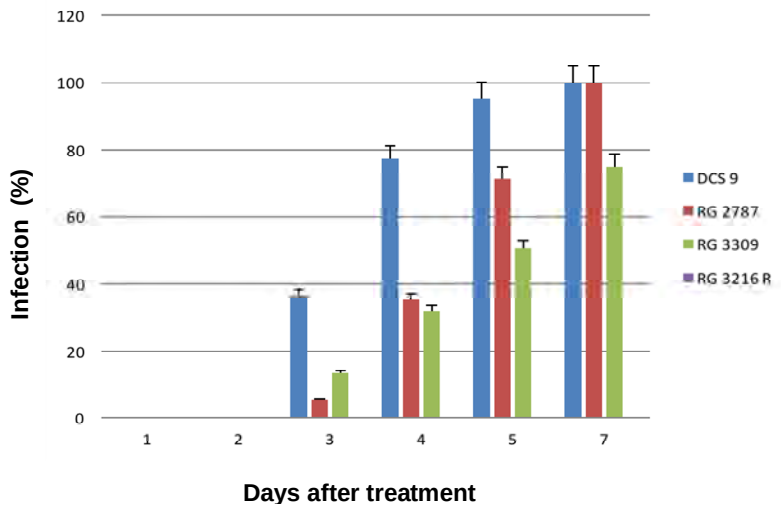

Fig. 2: Disease progression (infection %) of gray mold in castor genotypes; Error bars are indicated in graph. The values are mean of 2 replication data

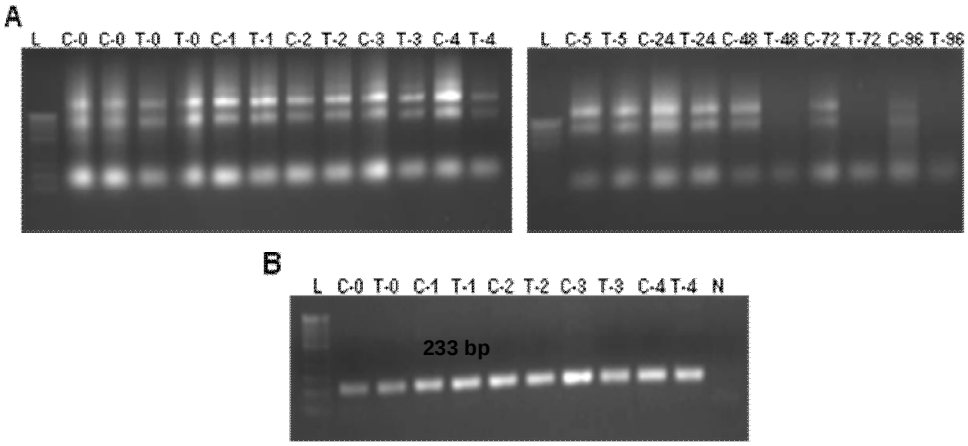

Fig. 3: Comparison of RNA integrity and *ubiquitin* (control) gene expression in control and treated samples of DCS 9 at various time intervals; A) Agarose gel electrophoresis of RNA from control (C) and treated (T) capsules till 4 days. Time intervals are denoted in number of hours post inoculation (numbers after hyphen), B) Expression of *ubiquitin* in control and treated samples till 4 hr. Lane L is 100 bp DNA ladder and N is negative water control.

Intactness of RNA and gene expression in infected samples

Intact and good quality RNA was isolated from treated capsule samples upto 24 hr after infection (Fig. 3A) or one day after treatment. Total RNA degradation in treated samples started by 2 days after treatment even before the symptoms of disease or infection were visible. Symptoms of infection on the other hand were visible only by 3rd day after treatment. The total RNA from control started degrading from 4th day, which may due to severing of capsules from plants. The expression of control gene till 4 hr from control and treated samples are shown in Fig. 3B. The candidate genes which are involved in the infection process or defense mechanisms at an early stage can be studied using the culture plate technique. The expression profile of differentially expressed genes identified in susceptible and resistant potato cultivars, involved in potato-*Phytophthora infestans* interaction were verified up to 65 hr after infection using *in vitro* raised plants (Orlowska *et al*., 2012). For gene expression studies or RNA-seq, mRNA was isolated 3 days post-inoculation (dpi), from *Botrytis*-infected tomato fruit and grape berries and at 2 dpi for lettuce leaves with similar symptoms of *Botrytis* infections, such as water-soaked lesions without extensive tissue maceration and with no or limited fungal sporulation (Blanco-Ulate *et al*., 2014). The culture plate method described here can be used for differential gene expression studies of candidate genes involved in early infection process of gray mold pathogen (*Amphobotrys ricini*) of castor.

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