JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2007. Vol. **1**(2), p. 153-156

Molecular Identification of a Fungus associated with the Holoparasitic Angiosperm *Hydnora abyssinica* in Namibia

Percy M. Chimwamurombe[1]*, Maass Erika[1] and Sheehama Jacob[2]

[1]Department of Biology, University of Namibia, P/Bag13301, Windhoek, Namibia.
[2]Department of Microbiology, Kazan State University, 420008, Kazan, Russia.

(Received: 05 August 2007; accepted: 12 September 2007)

In the present study, we have consistently observed a fungus that is in association with the fruits of the holoparasitic angiosperm *Hydnora abyssinica*. This holoparasite spends a large portion of its life cycle underground attached to the roots of its host (various *Acacia* species). This fungal-holoparasite association is intriguing in the sense that the fungus does not cause any reduction in seed viability of the holoparasite. In this study, the objective was to determine the identity of the fungus. Pure single spore cultures of the fungus were obtained and DNA was isolated from fungal mycelium to be used as templates in an internal transcribed spacer (ITS)-PCR amplification. The ITS products were electrophoresced on agarose gels and sequenced in an automated sequencer. Basic Local Alignment Search Tool (BLAST) searches revealed the identity of the fungus to be *Aspergillus niger*. We endeavour to determine the nature of the fungus –holoparasite interaction. We propose that the fungus helps in digesting the fruits to facilitate easy seed dispersal.

Key words: ITS, *Hydnora abssyinica*, holoparasite, *Aspergillus niger*.

Hydnora belongs to the family Hydnoraceae, a small family of angiospermous root parasites with only two genera, *Hydnora* and *Prosopanche*. While *Prosopanche* occurs exclusively in South and Central America[1], *Hydnora* is mainly an African genus of 4-5 species occurring in Africa, Madagascar and the southern tip of the Arabian Peninsula [2,3].

Hydnora is a poorly known genus with its center of diversity (*H. abyssinica, H. africana,* and *H. triceps*) the Karoo-Namib region of Namibia and the Richtersveld of South Africa. *Hydnora esculenta* is a very poorly collected member of the genus and is currently known only from the Berenty preserve in southern Madagascar as food for primates[4], while *H. sinandevu* was recently described from Tanzania[5].

* To whom all correspondence should be addressed.
Dr. Percy M. Chimwamurombe
Tel.: +264 61-2063358; Fax: +264 61-2063791
E-mail: pchimwa@unam.na

Hydnora abyssinica is known to parasitize the roots of mainly *Acacia* species such as *Acacia albida, A. cyanophilla, A. gerrardii, A. karroo, A. luderitzii, A. nilotica, A. seyal, A. tortilis.* [6, 7] and *A. newbrownii* (personal observation.). Of all the *Hydnora* species, this one has the greatest distribution and is found throughout Africa, from South Africa in the south to the Sudan and southern portions of the Arabian Peninsula in the north[8]. According to the latest review by Musselman & Visser, *H. johannis, H. solmsiana*, *H. cornii* Vacc., *H. ruspolii* Chiov., *H. bogosensis* Becc., and *H. hanningonii* Rendle are all synonyms for this taxon[6]. Although many publications use *H. johannis* as the preferred synonym, this publication will use *H. abyssinica* as the former appears to have priority over the latter - *Hydnora abyssinica* was first described in a flora of Ethiopia[9] in 1867, while the synonym *H. johannis* was only described four years later[10].

This species can be distinguished easily from other members of the genus because of its creamy-white, tetramerous flowers with an odor producing "cucculus" at the distal adaxial surface of the tepal – typical of pollinated by Coleoptera. The rhizome of *H. abyssinica* is terete while those of *H. africana* and *H. triceps* are terete to pentagonal[11].

In Namibia, *H. abyssinica* occurs primarily in savannah and woodland vegetation types as defined by Giess[12]. In the Namib Desert and karoo vegetation areas of Namibia and South Africa, its common hosts *A. karroo* and *A. newbrownii* occur mainly in river beds. Flowers are frequently observed only in disturbed habitats (personal observation).

The *H. abyssinica* fruit vary in size from 10 – 15 cm and is enclosed in a tough, dark brown pericarp. The pericarp is astringent in taste and surrounds a white, fleshy pulp with numerous strands of small brown seeds. The mature seeds are very hard, irregularly shaped and oblong to globose, 1 – 1.7 mm in length[6].

Fruits of *H. abyssinica* are often found in association with a fungus. Plant fungi can either be pathogenic or benign/symbiotic endophytes[13]. In cases where these are pathogens, they cause serious disease symptoms and eventually death; this can lead to economic losses as well as habitat loss which impact on biodiversity[14]. Fungal endophytes on the other hand are known to co-exist with the host plant without causing any disease symptoms[15] except in situations where the host plant is stressed[13].

The association between the fruit of *H. abysinnica* and the fungus is intriguing as it may play a role in the dispersal of the parasite seeds. The advent of the PCR technology has revolutionized the way of doing biological investigations. The PCR technique provides unprecedented sensitivity and it has facilitated development of a variety of nucleic acid based systems for diagnostic identification and genetic analysis of organisms[16]. Molecular identification of fungal species can now be done easily with great rapidity and accuracy by sequencing of the specific DNA regions like the internal transcribed spacer region (ITS) of the ribosomal DNA isolated from fungi.[16, 17, 18]

The present study aims to identify the fungus associated with the fruits of the angiospermous root parasite *H. abyssinica* by means of ITS sequencing and suggests a possible symbiotic relationship between this fungus and the parasite.

MATERIAL AND METHODS

Collection of *H. abyssinica* fruits

Fruits of the holoparasite *H. abyssinica* were collected from Okakeujo in the Etosha National Park, Northern Namibia in November 2005. These fruits were infected by a fungus which completely destroyed the fruit pulp, leaving only the seeds, covered in fungal spores.

Seed viability

Seeds were cleaned with a solution of 10% bleach to remove the fungal spores and rinsed three times with distilled water. Seeds were then divided into 5 replicates of 30 seeds and covered with distilled water in petri-dishes. The seeds were left for 24 hours at room temperature to imbibe water whereafter they were transferred to clean Petri-dishes containing 5 ml of a 0.5% triphenyl-tetrazoliumchloride (TTC) solution.

After 4 hours, all seeds were examined with a stereo microscope to determine their viability. Seeds that exhibited a distinct pink colour caused by the reduction of triphenyl-tetrazolium- chloride to triphenylformazan by the respiratory activities of the seeds were recorded as viable.

Isolation and identification of fungus

The fungus was cultured from the collected fruits. Single spore pure cultures of the fungus were grown on sterile 2% MEA media in the dark to mimic the dark conditions that host presents when it spend almost its entire life underground. It was grown for 10 days at 30°C. DNA was extracted from fungal mycelium using a Fermentas DNA isolation kit following the manufacturer's instructions.

PCR amplification of the ITS region of fungal DNA

The Go Green *Taq* Master Mix (Promega) which contains all ingredients needed for DNA amplification (dNTPs, *Taq* polymerase, $MgCl_2$, 10× PCR buffer) was used. The following reaction mixture was used: Go Green *Taq* Master Mix 12.5 µl, 5µM ITS1 primer 2 µl, ITS2 primer

2 µl, sterile double distilled water 4.5 µl and DNA template (10ng). The mixture was then put in the PCR machine under the PCR amplification profile: an initial of 94°C for 4 minutes followed by 32 cycles of 94°C at 30 seconds; 30 seconds at 62°C, and 1 minute at 72 °C then a final extension step of 10 minutes at 72 °C and holding at 4 °C. PCR products (5%) were analyzed on a 1% agarose gel stained with 0.5% ethidium bromide. The remainder of ITS PCR products were purified and sequenced by a commercial company in an automated sequencer using the Big-dye chemistry.

RESULTS

Seed viability

The TTC test revealed that almost 90% (87.8 + 2.2%) of the infected *H. abyssinica* seeds were viable.

Identification of fungus

DNA was isolated from 6 isolates of pure fungal cultures and ITS PCR products of about 400bp size were purified for sequencing. Sequences obtained for the 6 isolates were aligned and showed 100% similarity. Only one of the 6 sequences identical sequences was used to do nucleotide BLAST searches in the Genbank and the query fungal sequence understudy Accession number: bankit1013174 (EU126606) was shown to be 93 % similar to the *Aspergillus niger* sequence in the Genbank (accession number AY585555) (Fig. 1). We therefore conclude the identity of the fungus found in association with fruiting structures of *H. abyssinica* is A. *niger*. Morphological examinations on the isolated pure cultures supported the molecular ITS evidence.

```
Query     15   CCGTGTCGTATTCGTACCCTTGTTGCGTTCGGCGGGCCCGGCCGCATTGTCGGGCCGGCC   74
               ||||||| |||| ||| ||| ||||| |||||||||||| ||||| ||||| |||| |||
A.niger   46   CCGTGTC-TATT-GTAACCTGGTTGC-TTCGGCGGGCCCTGCCGC-TTGTC-GGCC-GCC   99
               ******* **** *** ********* ************ ***** ***** **** ***
Query     75   GGGGGGGCACCTCCTGCCCCCCGGGCCCGGGCCCAACAGGAGACCCCAACACGAACACTG   134
               |||||||| ||| ||||||||||||||| ||||| | ||||||||||||||||||||||
A.niger   100  GGGGGGGCGCCT-CTGCCCCCCGGGCCCGTGCCCAGCCGGAGACCCCAACACGAACACTG   158
               ******** *** *************** ***** * ********************
Query     135  TCTGAAAGCGTGCAGACTGAGTTGATTGAATGCAATCAGCTAAAACTTTCAACAATGGAT   194
               ||||||||||||||| |||||||||||||||||||||||| ||||||||||||||||||
A.niger   159  TCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGAT   218
               *************** ********************** ******************
Query     195  CTCTATG-TTCCAGCATCGAAGAAGAACGCAGC  226
               |||| || |||| ||||||| ||||||||||||
A.niger   219  CTCT-TGGTTCCGGCATCGATGAAGAACGCAGC  250
               **** ** **** ******* ************
```

Fig. 1. Local pairwise sequence alignment of ITS regions revealing similarity to *A. niger.* The asterisks indicate identity of bases. The ITS sequence of the fungus isolated in this study is indicated in grey.

DISCUSSION

We have successfully identified the fungus that has an association with the holoparasitic angiosperm, *H. abyssinica* to be *Aspergillus niger*. We have further demonstrated that infection of the fruit by the fungus does not negatively affect the viability of the seeds. The fungus thus does not seem to be pathogenic.

While more research is needed to establish the exact nature and incidence of this association, it is speculated that the fungus may aid in the dispersal of the *H. abyssinica* seeds. As the fruits are entirely subterranean, digestion of the fruit pulp by the fungus allows the large number of small seeds to blend with the soil in an area where host roots are plentiful, and the activity of insects and small mammals may further aid the dispersal of released seeds. The digestion is most likely by cell wall degrading enzymes secreted by the fungus.[19]

Being a holoparasite, the young *H. abyssinica* seedling cannot survive for a long period unless it attaches itself to the root of an

appropriate host. Digestion of the fruit pulp with the subsequent release of viable seeds in the vicinity of host roots may be an important dispersal mechanism that could significantly improve seedling establishment. Such a mechanism will concentrate seedlings in a relatively small area, something that is substantiated by field observations where heavy infestations are frequently observed. It should be noted that no record exist of a host plant that have been killed by *H. abyssinica*, in fact, field observations over many years suggest little, if any, damage to hosts. Fungus-aided dispersal may thus be an important dispersal mechanism in addition to rodents that will carry seeds further away from the parent plant.

REFERENCES

1. Cocucci, A. E and Cocucci, A.A. *Prosopanche* (Hydnoraceae): somatic and reproductive structures, biology, systematics, phylogeny and potentialities as a parasitic weed. In M.T. Moreno, J.I. Cubero, D. Berner, D. Joel, L.J. Musselman and C. Parker (Eds.). *Advances in Parasitic Plant Research.* 1996; 179 – 103.
2. Musselman, L.J. and McNeal, J. *Hydnora triceps* (Hydnoraceae): Unique flowers with an uncertain future. In Fer, A., Thalourn, P., Joel, D.M., Musselman, L.J., Parker, C. amd Verkleij, J.A.C. *Proceedings of the 7th International Parasitic Weed Symposium*. 2001; 23-28.
3. Musselman, L.J. and Visser, J.H. Taxonomy and natural history of *Hydnora* (Hydnoraceae). *Aliso,* 1989; **12**: 317 - 326.
4. Simmen, B., Hladik, A. and Ramasiarisoa, P. Food intake and dietary overlap in native *Lemur catta* and *Propothecus verreauxi* and the introduced *Eulemur fulvus* at Berenty, Southern Madagascar. *International Journal of Primatology.* 2003; **24:** 949-968.
5. Beentje, H. & Luke, Q. Flora of Tropical East Africa (2001).
6. Musselman, L.J. and Visser, J.H. *Hydnora johannis* in Southern Africa. *Dinteria*. 1987; **19**: 77 - 82.
7. Nyafuono, J.F., Odyek, O and Bukenya, R. Taxonomy and ethnobotany of *Hydnora* in Lake Mburo National Park (Uganda). *Israel Journal of Plant Sciences*. 2000; **48:** 99 - 103.
8. Visser, J.H. and Musselman, L.J. The strangest plant in the world. *Veld and Flora.* 1986; **71:** 109-111.
9. Braun, A. *Hydnora abyssinica* in Schweinfurth Beitrag zur Flora Aethiopiens, 1867.
10. Beccari, O Descizione die due nuove specie d'*Hydnora* l'*Abissinica*. *Nuovo Giornale Botanico Italiano* 1871; **3:** 6-7.
11. Musselman, LJ. The genus *Hydnora* (Hydnoraceaea). In: Ransom, KJ., Musselman LJ., Worsham AD and Parker C, eds. *Proceedings of the Fifth International Symposium on Parasitic Weeds*. Nairobi CIMMYT. 1991; 247-250.
12. Giess, W. A preliminary vegetation map of Namibia. Dinteria. 1971; **4:** 5-15.
13. Smith, H., Wingfield, M.J., Crous, P.W. and Coutinho, T.A. *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African Journal of Botany.* 1996; **62:** 86-88.
14. Crous, P.W., Groenewald, J.Z., Pongpanich, K., Himaman, W., Arzanlou, M. and Wingfield, M.J. Cryptic speciation and host specificity among *Mycosphaerella* spp. occuring on Australian *Acacia* species grown as exotics in the tropics. *Studies in Mycology.* 2004; **50:** 457-469.
15. Hunter, G.C., Roux, J., Wingfield, B.D., Crous, P.W. & Wingfield, M.J. *Mycosphaerella* species causing leaf disease in South African *Eucalyptus* plantations. *Mycological Research.* 2004; **108:** 1-10.
16. Wingfield, M.J., De Beer, C., Visser, C.D. and Wingfield, B.D. A new *Ceratocystis* species defined using morphological and ribosomal DNA comparisons. *Systematic and Applied Microbiology*. 1996; **19:** 191-202.
17. Vermeulen M, Roux J, Gryzenhout M, Chimwamurombe PM, and Wingfield MJ. A new report of *Chrysopoarthe austroafrican* occurring on Namibian *Syzgium guiniense* tress. *Southern African Society for Plant Pathologist* Abstracts, 2007.
18. Fouche N, J Roux, Solheim H, Heath R, Kamgan-Nkuekam G, Chimwamurombe PM, Pegg KG and Wingfield MJ. Identification of *Ceratocystis* species from hardword trees in Namibia, Australia and Norway. *Southern African Society for Plant Pathologist* Abstracts 2007.
19. Chimwamurombe PM, Wingfield, BD Botha A-M and Wingfield MJ. Production of polygalacturonases by isolates of *Cryphonectria cubensis* of differing virulence. *Biosciences, Biotechnology Research Asia*. 2005; **3**(2): 252-259.