INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print); 2222-5234 (Online)
Website: https://www.innspub.net
Email contact: info@innspub.net
Vol. 28, Issue: 6, p. 333-341, 2026

RESEARCH PAPER OPEN ACCESS

Identification and virulence of local strains of entomopathogenic fungi of the fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae) in the Niayes and Casamance regions of Senegal

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Key words: Virulence, Local strains, Entomopathogenic fungi, Fruit, *Bactrocera dorsalis*, Niayes, Casamance

Received: June 12, 2026 **Accepted:** June 26, 2026 **Published:** June 30, 2026

DOI: https://dx.doi.org/10.12692/ijb/28.6.333-341

ABSTRACT

Fruit production faces numerous threats from *Bactrocera dorsalis* fruit fly bites, which affect quality, yield and exports, and above all, chemical control problems caused by fruit flies in orchards. The aim of this study was to collect fly and soil samples, isolate and identify local strains of entomopathogenic fungi of *Bactrocera dorsalis*, and test their virulence for use in biological control. This study was conducted in the Niayes and Casamance regions of Senegal, where fly and soil samples were collected, wrapped and stored. Once in the laboratory, after washing, the fly samples were seeded in Petri dishes containing PDA or SDA, while the soil samples were mixed with distilled water before being seeded in dishes using beads. These seeded dishes were incubated in an oven. Macroscopic and microscopic identification of the fungi found in the flies showed the presence of *Fusarium* spp., *Aspergillus* spp., *Alternaria* spp., *Curvilaria* spp., *Pestalotia* sp., *and Colletotrichum* sp. Other local strains were isolated from the soil, including *Beltrania* sp., *Beauveria* spp., *Trichoderma* spp., *Rhysoctonia* sp., *Fusarium* sp., and *Aspergillus* sp., with a predominance of *Aspergillus* followed by *Fusarium* spp. and *Beauveria* spp. To determine the diversity of fungi in the two areas, the Shannon index was calculated and showed that the Niayes area is much more diverse than Casamance, with 1.961 versus 0.562 for flies and 1.934 versus 1.238 for soil. Virulence tests showed that the isolated entomopathogenic fungi had no effect on pupal mortality after 5, 10, and 15 days of observation.

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INTRODUCTION

In Senegal, the fruit industry is a promising sector with strong growth potential (Dieye, 2017). It is widely used by the local population. However, West African fruit industries have been facing the problem of fruit flies for many years. Fruit infestation rates vary between 5 and 100% in Africa, depending on location and season (Adebayo *et al.*, 2017). The introduction and spread of the fly, which has recently been described and is believed to be of Asian origin, is prompting all fruit growers to consider control measures due to the significant economic damage it causes (Ndoye, 2011).

It causes serious problems for international trade, particularly for agricultural raw materials in orchards (Papadopoulos and Quilici, 2014). In West Africa, the extent of damage caused by flies is increasing in orchards intended for export. In Senegal, mango production losses caused by *B. dorsalis* can be estimated at 40-60% because the peak in its fruit fly population coincides with the ripening of mangoes (CTA Practical Guides Collection, 2013). The population of this species increases considerably with the fruit harvest. Thus, the control of crop pests relies largely on the use of synthetic chemicals, which in many cases are used indiscriminately, complicating control efforts due to the disinfection treatments applied with harmful active ingredients (Diop, 2019).

This method remains ineffective because of the issue of pest adaptation. In addition, chemicals remain in the fruit, and residues remain in the soil and water. This causes enormous human health problems. In short, the unintended effects of these synthetic substances have led to alternative solutions, including the search for biological control agents (Kouadio *et al.*, 2018). Trials have long shown promising results with entomopathogenic fungi of the genera *Beauveria* and *Metarhizium*.

The overall objective is to contribute to the control of the fruit fly *B. dorsalis* in order to limit infestations and increase fruit production yields.

MATERIALS AND METHODS

Study area

An exploratory mission was carried out in the Casamance region, which comprises three departments: Kolda, Medina Yoro Foula and Vélingara. The Vélingara department is the largest, covering 40% of the region's surface area, followed by Medina Yoro Foula with 34% and Kolda with 26%. Soil samples were obtained in the Kolda region; samples were also taken along the Goudomp, Tanafe and Saré Yoba axis every 50 km travelled. Fly samples were only found in Vélingara.

The same was true in the Niayes region. However, the Niayes area has a fairly diversified horticultural production system and Guinean-type vegetation closely linked to soil type, topography and, above all, the presence of water in the environment. In addition, as a production system, the Niayes are a prime area for market gardening and fruit growing.

Sampling

An exploratory mission was carried out from 11 to 16 August 2020 in the Kolda and Ziguinchor regions. Eleven orchards were visited, including nine (9) old and two (2) young ones. Sampling was carried out along the Goudomp, Tanafe, Saré Yoba and Kolda axis every 50 km travelled. In these localities, a series of visits was carried out in six different orchards in the Niayes area from 24 to 25 August 2020. In these localities, the orchards were modern or old, young or unfenced, maintained or unmaintained. In the Niayes area, six (6) orchards were sampled, three (3) of which had fly samples. Sampling was simple random for the soil, which was collected from each mango tree in both the Niayes area and Casamance.

In Casamance, fly samples were only obtained in Bignona. The fly samples were collected in dry pheromone traps (sexual attractants), notably Methyl Eugenol (ME) and Malatrap. The attractants are placed inside the traps to attract only flies. The flies were then neutralised by dichlorvos, which was also placed in each trap. Empty 5-litre water bottles with holes in them and tied with wire were used as traditional yellow

traps, as shown in the images below. Each trap-attractant-insecticide unit, suspended from a tree branch, was numbered and georeferenced.

Preparation of the culture medium

For fungi, their culture media consist of PDA and SDA. Preparation is simple. First, for 1 litre of distilled water, thirty-five (35) g of PDA or 65 g of SDA were weighed, then mixed and homogenised using a stirrer. Once the bottle was completely homogenised, the mixture was placed in an autoclave for sterilisation. After removing the bottle and allowing it to cool slightly, the product was then poured into 9 cm diameter Petri dishes in the presence of a Bunsen burner flame, opening the dishes slightly to avoid contamination. Finally, the dishes that showed no contamination were used for seeding and isolating the fungi after 24 hours of observation.

Protocol for seeding and isolating fungi

Isolation from fly samples

The samples (flies) were first immersed in 1% bleach, then in 70% alcohol, then in sterile distilled water before being seeded in Petri dishes containing PDA or SDA medium. Seeding was carried out at a rate of 3 flies per dish near a flame.

On PDA medium, three flies were placed in Petri dishes and three replicates were made. The digestive tracts of the flies were then opened by pressing them with forceps and placed in Petri dishes containing PDA medium. A number of insects were placed in small tubes, crushed with brushes, then 3ml of distilled water was added and stirred thoroughly. A micropipette attached to a cone was used to pipette 100 µl of this solution (insects and distilled water) onto the PDA medium in the Petri dishes. Small beads were then used to spread the suspension until it was homogeneous. Finally, the beads were removed and the dishes were covered with parafilm.

Isolation from soil samples

This is the same method used with the crushed fly samples:

Soil samples (1 kg of soil per tree) also allowed us to search for entomopathogenic fungi. Soil samples were taken from under the mango trees at each location, and 3 g of soil was extracted from each sample and placed in tubes. Then 10 ml of distilled water was added. After shaking, the mixture was centrifuged to allow the fungal spores and other microorganisms to rise to the surface of the suspension. Thus, 100 µl of the supernatant was pipetted with a micropipette and seeded in a Petri dish containing PDA or SDA, then spread with beads.

The seeded Petri dishes were then closed and sealed with parafilm, labelled and incubated in an oven at 28°C. The label specifies the date and index corresponding to each sample. In order to obtain pure individual colonies, purification was carried out once the spores germinated and developed mycelium in the Petri dishes. This involved cutting out part of the mycelium in the growth areas with a sterile scalpel under aseptic conditions, taking a small amount of PDA, and transferring it to the middle of another Petri dish containing PDA. The transferred dishes were then incubated for 1 to 2 weeks for sporulation.

Identification of entomopathogenic fungi

Macroscopic and microscopic observation enabled us to identify the fungi. Macroscopic identification was done with the naked eye. We took into account the colour, appearance and density of the mycelium on the surface and underside of the Petri dishes, while microscopic identification was based on the shape of the spores and the appearance of the mycelium, using the identification keys of Barnet and Hunter and Tsuneo Watanabe. For microscopic observations, a small sample was taken from the 2- to 3-week-old mycelium, spread on a slide with 2 drops of distilled water, covered with a coverslip, and then observed.

The Shannon index allows us to determine the relative abundance of each isolated species as well as the specific diversity of each region. In our study, the two areas to be compared are the Niayes and Casamance regions.

$$H' = -\sum_{i=1}^{S} pi.log_2(pi)$$

Where,

H' = H' corresponds to the Shannon index.

p_i = the proportional abundance or percentage abundance of a species present ($p_i = N_i/N$).

N_i = the number of individuals counted for a species present

The relative abundance of fungi was also calculated, which was:

Ar= (Aa*100)/N

Where,

Ar = abondance relative

Aa = abondance absolue where Aa is the number of individuals of a species

The percentage of pupal mortality was also studied, which is simply

Mortality rate = (NPD/N) ×100

Where,

NPD: number of dead pupae

N: total number of puppies

In vitro virulence test on *Bactrocera dorsalis* species using entomopathogenic fungi (Ziani, 2008). First, a suspension of entomopathogenic fungal spores had to be prepared, which consisted of mixing 1 ml of Tween and 9 ml of distilled water and pouring it over the isolate of each fungus, scraping it and sieving it to recover the suspension. This mixture was then used to immerse *Bactrocera dorsalis* pupae, which were then placed in sterile soil. Finally, the pupae were incubated to observe their effect and record their mortality rates.

Data analysis

The analysis of inhibition data and fly mortality during tests with entomopathogenic fungi was made possible using R 3.2.1 software (R Core Team, 2016). A frequency analysis was performed using the Fisher function. Test for comparing fungal distribution. Fungal biodiversity in the two areas was studied using the Shannon index. Other data were processed using Microsoft Office Excel 2013.

RESULTS

The entomopathogenic fungi investigated in this study are *Beauveria* sp., *Metarhizium* sp. and *Fusarium* sp., which effectively control insects. However, only Beauveria and Fusarium fungi were isolated from the soil.

Table 1 shows the fungi isolated from the flies. From this table, the Mbidium orchard has many more species than the other orchards in the Niaye zone, namely Ngadiaga, Ndam Lo and Casamance (Bignonna). *Aspergillus* spp. is present in all orchards, while *Fusarium* spp. species are only present in the orchards of Ndame Lo and Bignonna. *Phytophtora* sp. is found in Mbidieum and Ngadiaga. *Alternaria* spp., *Pestalotia* sp., *Curvilaria* sp., and *Lasidiodiplodia* sp. are only present in the Mbidium orchard.

Table 2 shows the species of fungi isolated from the soil in both areas. *Beauveria* spp. and Aspergillus spp. were present in almost all orchards, both in the Niayes area and in Casamance.

Fusarium spp. is present in Casamance in the orchards of Philomène, Yoro Walo, Assane Touré Kounkané, and Ndamlo in the Niayes area. Other species were also isolated: *Beltrania* sp. (Diop Sao), *Pythium* spp. (Ndamlo, Mboro), *Trichoderma* spp. (Yoro Walo, Mama Aly, Saré Yoba, Ndamlo), *Rhysoctonia* sp. (GD) and *Phoma* spp. (Ndie, Nga).

Abundance of isolated fungi

Fig. 1 shows the frequency of fungi isolated from flies in the six orchards in the Niayes area and the Teubi orchard in Casamance. Analysis of this Fig. 1 shows the dominance of *Aspergillus* sp. (39%), followed by *Fusarium* spp. (25%) and *Colletotrichum* sp. (11%), and finally *Phytophtora* spp. *Curvularia* sp. and *Alternaria* sp. each account for 7%. In addition, 14% of non-spore-forming fungi were also isolated. Thus, in Casamance, *Aspergillus* sp. dominates with 75% compared to *Fusarium* sp., which represents 25%.

Table 1. Fungi isolated from flies

Champignons	Mbidium	Bignona	Ngadiaga	Ndame Lo
Fusarium spp.		++		++
Aspergillus spp.	++	++	++	++
Alternaria spp.	++			
Pestalotia sp.	++			
Phytophtora spp.	++		++	
Curvilaria sp.	++			
Colletotrichum sp.	++			
Lasidiodyplodia sp.	++			

++: signifies the presence of the species

Table 2. Fungi isolated from soil

Localités/ Champignons	Zones													
	Casamance									Niayes				
	Phil	Sar sa	GD	Yoro walo	Ma Aly	Ass kou	Kolda	Sar Yob	Ndie	Diop Sao	Nd	Nga	mboro	mbi
Beltrania sp.										++				
Fusarium sp.	++			++		++					++			
Pythium sp.										++	++			
Trichoderma sp.				++	++			++			++			
Aspergillus sp.	++	++	++	++	++	++		++		++		++	++	++
Rhysoctonia sp.			++											
Beauveria sp.	++	++	++		++		++			++		++	++	
Phoma sp.									++			++		
Champignons non identifiés				++										++

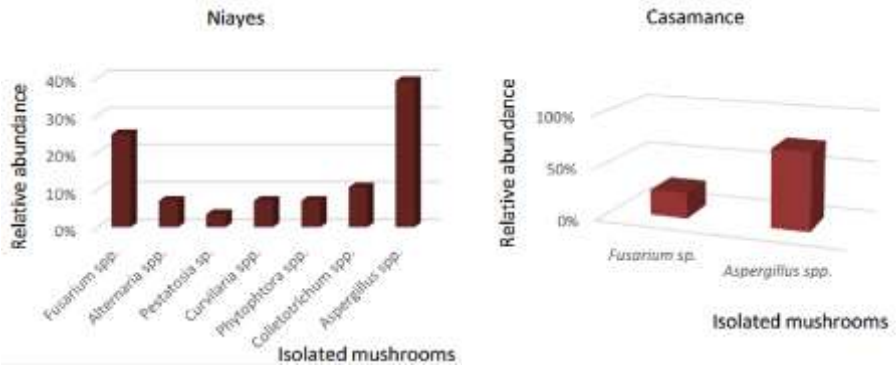

Fig. 1. Abundance of mushrooms isolated from flies in the Niayes area and in Casamance

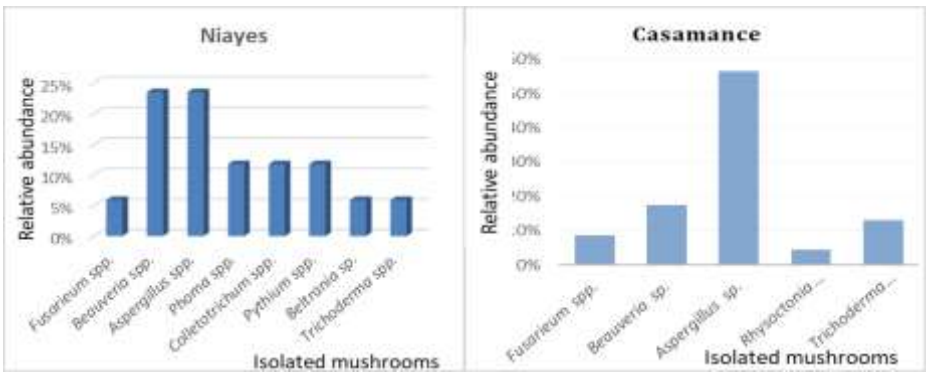

Fig. 2. Abundance of mushrooms isolated from flies in the Niayes area and in Casamance

Fig. 2 shows the frequency of each fungus isolated from the soil in the Niayes area and also in Casamance. The graph for the Niayes area shows that *Beauveria* sp. and *Aspergillus* sp. have the same proportion (24%), followed by *Phoma* sp., *Colletotrichum* sp. and *Pythium* sp., which also have the same relative abundance (12%), and finally *Trichoderma* sp., *Beltrania* sp. and *Fusarium* sp., which have the smallest proportion, each with 6%. Furthermore, in Casamance, analysis of the graph shows the dominance of *Aspergillus sp*. with 57%, followed by *Beauveria* sp. (17%), *Trichoderma* sp. (13%), *Fusarium* sp. (9%) and *Rhysoctonia* sp., which has the smallest proportion (4%).

Mushroom diversity

It was therefore deemed necessary to determine the diversity of species using the Shannon index for each locality and to compare them. For the Niayes area, the Shannon index was 1.961 compared to 0.562 in Casamance, showing that the Niayes area is much more diverse in terms of mushroom species than Casamance. The Shannon index was also calculated in these areas, with the Niayes area having an index of 1.934 compared to 1.238 in Casamance. This also shows that the Niayes area has many more fungi than Casamance.

Characterisation of entomopathogenic fungi

Only two of the fungi were represented in this article, namely *Beauveria* sp. and *Fusarium* sp. The appearance of the mycelium of *Fusarium* sp. 1 is white to yellowish, sometimes concentric in shape. The mycelium is cottony. The underside of the box is yellow in colour. The mycelium fills the box after 5 to 7 days of incubation. Microscopic examination reveals short, long phialides formed on the aerial mycelium. The macroconidia are curved, fusiform and septate (Fig. 3).

Front view Behind view Microscopic appearance

Fig. 3. *Fusarium* sp. isolates on PDA medium isolated from ndamlo flies (magnification: 400)

Front view Behind view Microscopic appearance

Fig. 4. *Beauveria* sp. 6 isolated from soil on SDA medium at Philomène (magnification: x 1000)

Beauveria sp. 6 was isolated with a white, cottony and very dense mycelium with a white to yellowish reverse. The mycelium filled the box after 15 days. The microscopic appearance of this species showed many small spherical spores with conidiophores in the form of clusters (Fig. 4).

***In vitro* virulence tests of entomopathogenic fungi**

The entomopathogenic fungi that were tested were species of the genus *Beauveria* from Ngadiaga, Goudomp (vehlingara), Philomène and a strain of *Fusarium* sp. from Ndamlo. Not all species could be

tested due to the unavailability of pupae. Analyses were carried out to assess the abortion of certain pupae that had not emerged after 5 days, 10 days and 15 days. These had *p*-values of 0.5217, 0.9774 and 0.8952 respectively, which were not significant. This shows that the fungi tested had no effect on the pupae. Table 3 shows the average percentages of pupae that did not emerge for each fungus tested after 5, 10 and 15 days.

Table 3. Average percentages of pupal abortion

Treatments	FNE 5 (%)	FNE 10 (%)	FNE 15 (%)
T	70	70	70
Ba	83	80	80
Bb	80	76	76
Bc	70	70	66
F	76	70	70

ba: *Beauveria* Ngadiaga, bb: *Beauveria* Goudomp, bc: *Beauveria* philomene, t: témoins, f: *Fusarium* sp, FNE: non-emerged fly 5 days, 10 days and 15 days

DISCUSSION

Fruit production faces numerous threats from *Bactrocera dorsalis* (Hendel) which, according to Ndiaye (2008), is present in large numbers on several fruit species, particularly mangoes. Work to isolate and identify fungi in fly samples from orchards surveyed in the Niayes region revealed a high level of diversity. The fungi isolated, in order of importance, are as follows: *Aspergillus* spp. (39%), *Fusarium* spp. (25%), *Colletotrichum* sp. (11%), *Alternaria* spp. (7%) and *Pestalotia* sp. 4%. In Casamance, in the Teubi orchard, only *Aspergillus* spp. (75%) and *Fusarium* sp. (25%) were isolated. The Shannon indices calculated in the Niayes and Casamance areas (1.961 and 0.562, respectively) showed that fungal diversity is much greater in the Niayes orchards than in Casamance. This fly-fungus association includes entomopathogens that could cause their death.

This corroborates the work of Farida *et al.* (2011), which showed that *Fusarium* sp., *Alternaria* sp. *and Aspergillus* sp. found in Phyllocnistis citrella larvae could cause their death.

Furthermore, the genus *Beauveria* was not isolated from the flies. This is thought to be due to the high humidity in the plantations. According to Aregger (1992), high soil moisture reduces the viability of Beauveria spores. Isolation work from soil samples in the Niayes and Casamance orchards also showed a diversity of fungi, notably *Fusarium* spp, *Beauveria* spp., *Aspergillus* spp., *Trichoderma* spp., *Colletotrichum* sp., *Rhysoctonia* sp. and *Beltrania* sp. The Shannon indices calculated for this method also showed that the Niayes area has more species, with 1.934 compared to 1.238 in Casamance. It is therefore the most diverse. However, entomopathogenic fungi of the genus *Fusarium* and *Beauveria* have been found in some orchards in Casamance and Niayes. The presence of these species in the soil could be favoured by soil moisture. In Casamance, soil moisture is very high during the rainy season, particularly in orchards where entomopathogenic fungi of the genus *Beauveria* have been isolated. These fungi naturally infect host insects. The virulence tests of entomopathogenic species were chosen because of their abundance of spores, namely *Beauveria* spp. from Ngadiaga, Goudomp, Philomène and *Fusarium* sp. from Ndamlo. It is recognised that the mortality of insects infected by entomopathogenic fungi depends on the number of spores that come into contact with the insect (Badaoui, 2018). The results of this study compared to the untreated control showed insignificant differences in pupal mortality (p-value: 0.5217, 0.9774 and 0.8952) during the following 5, 10 and 15 days, respectively. This shows that the fungi *Fusarium* sp. 3 from Ndamlo, *Beauveria* sp. 1 from Ngadiaga, *Beauveria* sp. 4 from Goudomp, and *Beauveria* sp. 6 from Phylomène tested have no effect on pupae, which may be due in part to the pupae's integument, which is too hard to allow the mycelium to penetrate inside the pupae. Pathogenicity tests conducted by Bakelli and Habibi (2019) demonstrated the effectiveness of two indigenous entomopathogenic fungi, *Beauveria* sp. and *Fusarium* sp., against white grub larvae. Climatic factors can be extremely limiting in the development of entomomycoses and, in this case, present a real obstacle to their use in microbiological control, particularly in the context of sustainable

agricultural development (Vidal and Jaber, 2015). However, studies have shown that *Beauveria bassiana* can infect the host through direct contact or ingestion (de Kouassi, 2001). Thus, the work of Mehinto *et al.* (2015) reported mortality rates ranging from 30 to 100% in 10 days of incubation of cowpea pest insect larvae inoculated with spores of *B. bassiana* and *Metarhizium anisopliae*. Similarly, the work of Sabbahi *et al.* (2008) showed high mortality rates among strawberry weevils due to the entomopathogenic fungi *Beauveria bassiana*.

CONCLUSION

Isolation and identification work revealed the presence of various fungi associated with the flies: *Fusarium* spp., *Aspergillus* sp., *Curvilaria spp*., *Pestalotia* sp., *Colletotrichum* sp. At ground level, *Beltrania* sp., *Beauveria* spp., *Trichoderma* spp., *Rhysoctonia* sp., *Fusarium* sp. and *Aspergillus* sp. Shannon indices showed that the Niayes area is more diverse than Casamance in terms of fungal species. The study showed that Fusarium species found in flies could well cause the death of these species. Another species of vital importance for sustainable biological control was also isolated from the soil, *Beauveria* sp., in certain orchards in the Niayes and Casamance regions. However, this species of *Beauveria sp*. could not be isolated in flies. Virulence tests carried out on the entomopathogenic fungi *Beauveria sp*. 1, *Beauveria* sp. 5, *Beauveria* sp. 8 and *Fusarium* sp. 3 unfortunately had no effect on *B. dorsalis* pupae.

RECOMMENDATIONS

The findings of this study lead to several recommendations for future research. The various entomopathogenic fungi identified should be characterized at the molecular level to accurately determine their species. Further studies are also needed to improve understanding of the ecology and persistence of *Beauveria* sp. under field conditions. In addition, the virulence of these fungi should be evaluated against third-instar (L3) larvae and adult stages of *Bactrocera dorsalis* to assess their potential as effective biological control agents.

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