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Research Article

Fungi Associated with Seedling Diseases of *Eucalyptus saligna* and *Prunus africana* in Cameroon and Chemical Control of Cercospora Leaf Spot

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ABSTRACT

Seedlings diseases are one of the main biotic constraints in seedling production of *Eucalyptus saligna* and *Prunus africana* in Cameroon. Unfortunately, these diseases are not yet identified. The aim of this study was to contribute to the improvement of production of healthy seedlings of *E. saligna* and *P. africana*. To achieve this, infected leaves of the two seedlings were collected and fungi isolated on potato dextrose agar (PDA) medium, then identified based on their morphological and microscopic characteristics by using reference documents. Antifungal activity of the following synthetic fungicides: Metalaxyl (8%) + Mancozeb (64%) active ingredient (a.i.) of Fongistar 72% WP, Prothioconazole (250 g/l) a.i. of Fongipro WG, Chlorothalonil (400 g/kg) + Dimethomorph (80 g/kg) a.i. of Sphinx 480 WDG and Cupper oxyde (86%) a.i. of Nordox 75 WG was tested *in vitro* at 0.5, 1 and 2 mg/ml on the growth of *Cercospora sp* on PDA and *in plantae* at prescribed doses by the manufacturer. Results show that seedlings of *E. saligna* and *P. africana* are infected by various fungi of phytopathological importance, the most common being *Cercospora sp* (25% and 27.8% in *E. saligna* and *P. africana* respectively) followed by *Aspergillus niger* and *Rhizoctonia* sp. *In vitro* tests reveal that fungicide formulations Fongipro WG and Sphinx 480 WDG at 2 mg/ml had the highest significant ($P<0.05$) percent inhibition (94.12 and 100% respectively), and the lowest EC_{50} and EC_{90} compared to other fungicides. Seedlings treated with Fongipro WG, Fongistar 72% WP and Sphinx 480 WDG gave the lowest disease incidence and severity on seedlings of the two plant species. Seedlings of *P. africana* were more susceptible to Cercospora leaf spot compared to *E. saligna* in the study area. The study shows that Cercospora leaf spot is the main foliar disease of *E. saligna* and *P. africana* seedlings and fungicides Fongipro WG, Fongistar 72% WP or Sphinx 480 WDG could be suitable for homologation against Cercospora leaf spot of both species in the nursery.

Key words: Cercospora leaf spot, Disease incidence and severity, Eucalyptus, Pygeum, Synthetic fungicides.

INTRODUCTION

The Congo basin is considered the second largest forest in the humid tropical zone after the Amazon basin, for its wealth of natural resources and biodiversity. Cameroon is one of the important components of this forest zone with about 20 million hectares of tropical rainforest (MINFOF, 2007). According to the World Conservation Monitoring Center (WCMC), in Cameroon, the main important woody forest resources are overexploited for commercial or domestic purposes and must constantly be planted in order to limit the harmful effects on the forest, human and biosphere (WCMC, 1996). Thus, in its reforestation policy, the Cameroon government through the National Forestry Development Agency (ANAFOR) has the mission of ensuring forest regeneration in several areas in Cameroon. In the ANAFOR station of Dschang city, the main seedlings produced and marketed are *Eucalyptus saligna* and *Prunus africana. E. saligna* is an exotic species that significant progress in the fields of forest genetics and tree physiology, but also in terms of forestry and wood technology (furniture manufacturing, structural construction and medicinal use). This fast-growing woody

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tree species adapts to a variety of ecosystems. In Cameroon, *E. saligna* dominates the landscape of the Northwest and Western regions where its introduction dates back to the 1920s (Pouomogne, 1983; Mbah, 1990). Eucalyptus fiber is now recognized as the international standard for short fiber and has penetrated all markets and for that reason, it has become a strategic tree, a new "green gold" (Foelkel, 1998). On the other side, *Prunus africana*, with the commercial name pygeum, is a forest species valued and planted for the medicinal properties of its bark (treatment of prostate cancer) and the quality of its wood which is highly valued in the international market used mainly for the manufacture of furniture (Njamnshi and Ekati, 2008). However, in Cameroon, *P. africana* is nowadays listed as a plant species threatened with extinction by the International Union for the Conservation of Nature (IUCN) which led to its classification in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Belinga, 2011). Despite the socioeconomic importance of these two forest species, in Cameroon their production is confronted with several factors. Among these factors, the indiscriminate exploitation of their resources and the application of unsustainable harvesting methods (Belinga, 2011), diseases that affect seedlings and the products after harvesting which reduce the wood quality and therefore their added value. In some cases, post-harvest losses can be greater than 40% of potential production (Singunda, 2009). It is estimated that in forestry nurseries, 10% of potential global production is lost due to seedling diseases (Larignon *et al.*, 2005). In Cameroon, seedlings diseases of *E. saligna* and *P. africana* are the main biological constraints that hinder seedlings production in forestry nurseries and later in plantations. Unfortunately, these diseases are not yet identified. Thus, the objective of this study was to improve the production of Eucalyptus and Pygeum plants through identification of fungi associated with seedling diseases and the control of Cercospora leaf spot using synthetic fungicides.

MATERIALS AND METHODS

Isolation and identification of fungi associated with *E. saligna* and *P. africana* seedlings

Infected leaves of *E. saligna* and *P. africana* were collected at the experimental nursery of the ANAFOR station in Dschang during the months of July and August 2016 and transported to the Phytopathology Lab for culture, isolation and identification. Culture and isolation of fungi were carried out on potato dextrose agar (PDA) medium and incubated in the dark at 21±1°C following the procedure described by Djeugap *et al.* (2015) and Keuete *et al.* (2015). Fungal identification was done under an ordinary microscope (Olympus BH_2) using identification keys in mycology (Barnett and Hunter, 1972; Mathur and Olga, 2003; Warharm *et al.,* 2008). During fungal purification and isolation steps, isolation frequency (IF) of each fungus were calculated using the following formula IF = (NF/NT) x 100, where NF is the total higher number of samples from which a particular fungus was isolated and NT is the total number of samples from which isolations were carried out (Iqbal and Saeed, 2012).

In vitro evaluation of the antifungal activity of synthetic fungicides on the development of *Cercospora sp*

The evaluation of the *in vitro* activity of fungicides was achieved by measuring the inhibition percent of radial growth of *Cercospora* sp by four formulations of synthetic fungicides (Fongistar 72% WP, Fongipro WG, Nordox 75 WG and Sphinx 480 WDG) through the agar dilution method (Sharma and Trivedi, 2002). Fungicides were tested at concentrations of 0.5; 1 and 2 mg/ml. These concentrations were obtained by adding 1 ml of each of the fungicide dilutions previously prepared in 19 ml of the PDA medium at 40ºC (Keuete *et al.,* 2015). After homogenization using a vortex, the medium was poured into Petri dishes (90 mm in diameter). In control Petri dishes, fungicides were replaced with 1 ml of distilled water. After solidification of the medium, a mycelium explant (5mm in diameter) was removed from the growth front of 7 days-old pure culture of *Cercospora sp* using a cookie cutter of 5mm in diameter, then deposited aseptically in the middle of each Petri dish supplemented with fungicides or distilled water (control). These Petri dishes were then sealed with parafilm paper and incubated at 21 ± 1°C for 7 days (time required for the fungal colony in control Petri dishes to totally colonize the Petri dishes). The percentage inhibition (PI) of the growth of *Cercospora sp* by the different fungicides was obtained as follows: PI = 100 x (DT– D) /DT (Djeugap *et al*., 2011). Where DT is the growth diameter of pathogen in the control Petri dishes and D the growth diameter of the pathogen in the Petri dishes supplemented with the fungicides. The *in vitro* test was done in a completely randomized design with 3 replications. The equivalent concentration of 50% (EC_{50}) and 90% (EC_{90}) of the inhibition percent of the fungal growth were obtained by transforming inhibition percentages into probits prior to statistical analysis. The characteristics of the fungicides used are presented in Table 1.

Seedling production and *in vivo* evaluation of the effect of fungicide on Cercospora leaf spot of Eucalyptus and Pygeum seedlings

One kilogram of healthy seeds of each plant species was separately introduced into a 10 liter bucket with water (for flotation test). All floating seeds were removed and the dense seeds were sown in propagation tray. A few weeks after germination, the healthy seedlings were

Table 1: Active ingredient, mode of action and dose of the synthetic fungicides used in the experiment.

Trade name	Active (s) ingredient (s)	Mode of action	Dose (mg/ml) and frequency of treatments
Fongistar 72% WP	Metalaxyl (8%) and Mancozeb (64%)	Systemic and contact	333 mg/ml, spray every 21 days
Sphinx 480WDG	Chlorothalonil (400 g/kg) and Dimethomorph (80 g/kg)	Systemic and contact	4 mg/ml, spray every 21 days
Nordox 75WG	Copper oxide (86%)	Contact	2.67 mg/ml, spray every 15 days
Fongipro WG	Prothioconazole (250 g/l)	Systemic	3.33 mg/ml, spray every 21 days

thoroughly sprinkled with water and gently pulled out to be transplanted into 20 cm diameter polyethylene bags containing black soil and poultry manure in the ratio of 3:1 where they continued to grow. Seedlings were sprayed with the following fungicide formulations: Fongistar 72% WP, Fongipro WG, Nordox 75 WG and Sphinx 480 WDG once a week during one month according to the manufacturer's recommendation. Control seedlings were sprayed with distilled water at the same rate. Each fungicide was sprayed on 10 seedlings of each plant species and three replications were considered. So, 450 plants were used per species during experimentation. Cercospora leaf spot incidence (I) and severity were recorded every week, before spraying as follow: I (%) = (number of leaves infected on the seedlings/Total number of leaves on the seedlings) x 100 (Djeugap *et al*., 2014). Severity was determined using the modified scale of disease measurement developed by Horsfall and Barrat (Berger, 1980).

Statistical analysis

Data collected on foliar incidence and severity of Cercospora leaf spot, isolation frequency of fungi, percentage inhibition, EC_{50} and EC_{90} were subjected to analysis of variance (ANOVA) and means were separated by the Duncan Multiple test at the 5% using the SAS version 9.3 software.

RESULTS

Identification of fungi associated with seedlings of Eucalyptus and Pygeum and their morphological characteristics

Six fungal species identified were associated with seedlings of *Eucalyptus saligna* and seven species with *Prunus africana*. Among these species, five were isolated from seedlings of the two plant species (Table 2). The most frequent fungus was *Cercospora sp* with an isolation frequency of 27.78% and 25% in *E. saligna* and *P. africana* seedlings respectively. The least common fungal species was *Trichoderma harzianum* (5.56%) in *E. saligna* and *Cladosporium sp* (6.25%) in *P. africana* (Table 2). Figure 1 presents the morphocultural characteristics of some fungi isolated on the two plant species. Mycelium of all the species is septate; their physical aspect/morphology varies from one species to another. In fact, on PDA medium, the mycelium (14-days old culture) is dense in *Cercospora* sp, *Rhizoctonia* sp and *Verticillium* sp; black in *Aspergillus niger*, grey in *Cercospora* sp, whitish in *Pestalotiopsis* sp and brown in *Verticillium* sp. The conidia are very minute and abundant in *A. niger* and *Verticillium* sp while they are relatively large in size in *Pestalotiopsis* sp and *Cladosporium* sp. Mycelium of *Rhizoctonia* sp don’t produce spore (imperfect fungi) (Figure 1).

In vitro* effect of synthetic fungicides on radial growth of *Cercospora sp

Table 3 shows the effect of fungicides on growth inhibition of *Cercospora sp*. All the fungicides inhibited the growth of the pathogen at various degrees compared to control. Fungicide Sphinx 480 WDG, significantly inhibited the growth of *Cercospora sp* at all concentrations tested compared to others fungicides. Total inhibition (100%) was obtained at 2 mg/ml with this fungicide. However, at that concentration the inhibition was significantly similar to that of fungicide Fongipro WG (94.12%). Fungicide Nordox was the least efficient at all the concentrations tested. EC_{50} and EC_{90} were lower with Sphinx 480 WGD (0.49 and 0.83 mg/ml, respectively) compared to other fungicides. This corroborated *in vitro* result and confirms the efficiency of this fungicide in the control of *Cercospora* sp. In contrast, Nordox 75 WG was once more the least effective fungicide with the highest EC_{50} and EC_{90} values (Table 4).

Table 2: Isolation frequency (%) of fungi associated with diseases on *Eucalyptus saligna* and *Prunus africana* seedlings.

Fungal species	*E. saligna*	*P. africana*
Aspergillus niger	23.27	17.85
Cercospora sp	27.78	25
Cladosporium sp	0	6.25
Pestalotiopsis sp	11.11	12.5
Rhizoctonia sp	21.17	19.63
Trichoderma harzianum	5.56	0
Verticillium sp	11.11	18.75

***In vivo* effect of fungicides on Cercospora leaf spot of *E. saligna* and *P. africana* seedlings**

It was observed that seedlings of both tree species develop Cercospora leaf spot after fungicide application with variable intensity and severity with respect to the type of fungicides applied. Cercospora leaf spot incidence on both seedling species was significantly higher ($P<0.05$) on seedlings treated with water (control) and Nordox 75 WG compared to other treatments. Disease incidence and severity on seedlings treated with Fongipro, Fongistar and Sphinx were significantly similar with the lowest values compared to control and Nordox at the recommended dose (Table 5). Seedlings of *P. africana* were more susceptible to Cercospora leaf spot compared to *E. saligna*. In fact, disease incidence and severity were 78.7 and 54.3% on untreated seedlings of *E. saligna* and 92.4 and 66.3% on untreated seedlings of *P. africana*, respectively.

DISCUSSION

The study highlighted the existence of many fungi associated with infected seedlings of *E. saligna* and *P. africana*. Most of the identified fungi have been previously reported by several studies (Mwanza and Waithaka, 2001). *Cercospora sp* was the most frequent fungal species in both *P. africana* and *E. saligna*. This high frequency could be due to the fact that *P. africana* and *E. saligna* are endowed with organic substances much in demand by species of the genus Cercospora. According to (Regnier *et al.*, 2010), the high frequency of *Cercospora sp* is due to their presence in the seeds of these two species and would have been transmitted to the seedlings. In addition, Cercospora sp is a polyphagous species capable of developing on several plant species such as: *Pericopsis elata* and *Persea americana* (ITB, 2009; Djeugap *et al.*, 2015; Djeugap *et al.*, 2017). However, species such as *Aspergillus niger*, *Rhizoctonia solani*, *Pestalotiopsis sp* and *Verticillium sp* were the least frequent on seedlings. This low isolation frequency could be of these due to the fact that they colonize the outer part

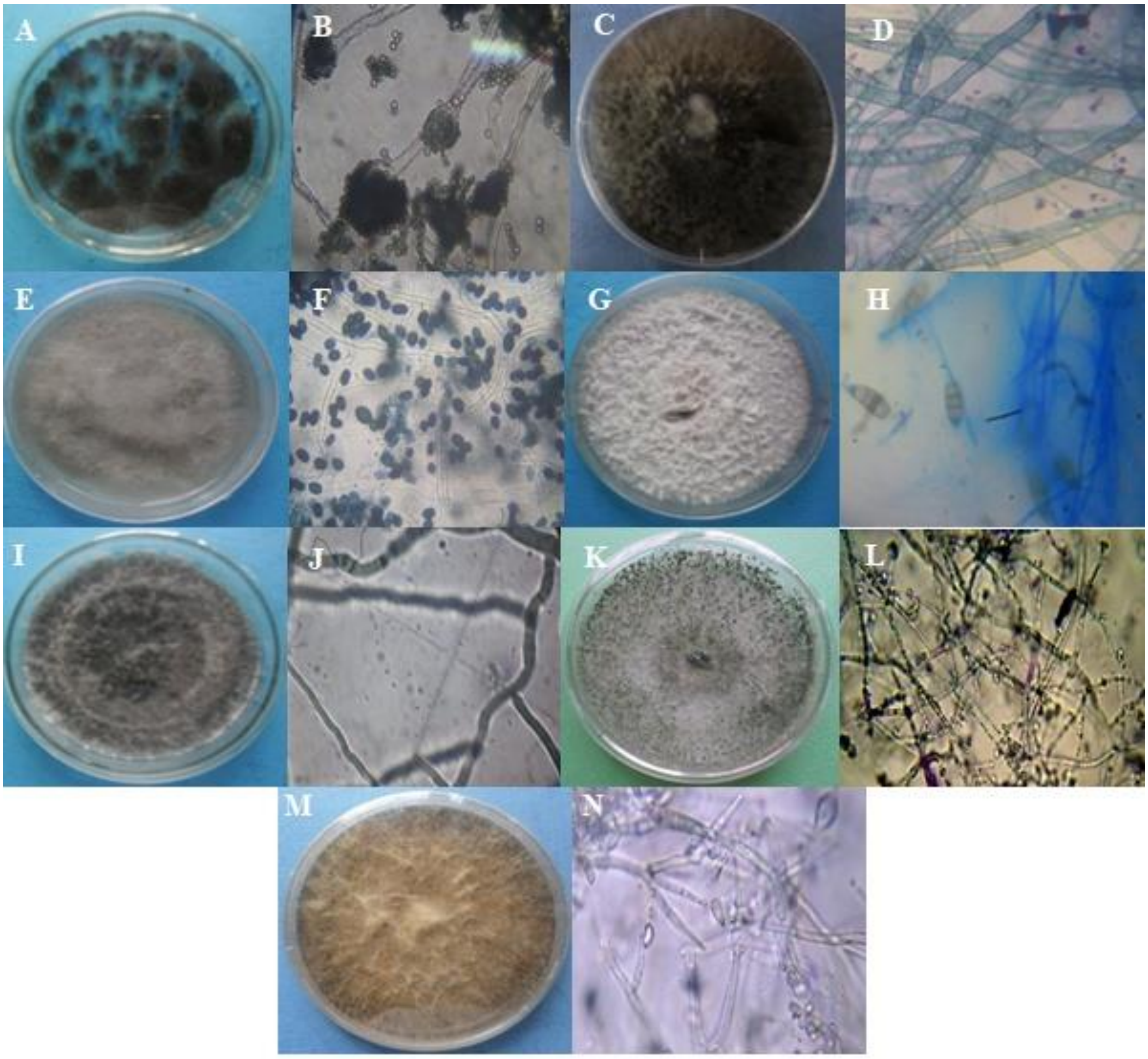

Fig. 1: 14-days old of pure culture and microscopic morphology (mycelium and conidia) of some fungi isolated on seedlings of *Eucalyptus saligna* and *Prunus africana* leaves. A and B: *Aspergillus niger,* C and D: *Cercospora* sp, E and F: *Cladosporium* sp, G and H: *Pestalotiopsis* sp, I and J: *Rhizoctonia* sp, K and L: *Trichoderma harzianum*, M and N: *Verticillium* sp.

(superficial fungi or opportunistic fungi) of the lesion and therefore were destroyed by the disinfectant solution (bleach) while the pathogen is deeper in infected leaf tissues.

In vitro antifungal activity showed that the all the chemical fungicides tested had a depressive effect on the radial growth of *Cercospora sp*. The radial growth of *Cercospora sp* was influenced by concentrations as well as by the type of fungicide used. The radial growth inhibition of the pathogen by these fungicides could be attributed to their active ingredients which mode of action were either contact, systemic or both (Zwieten *et al.*, 2004). Radial growth of the pathogen decreased with increasing concentration of fungicide. Similar results have been reported by Everett *et al.* (2007), who found that synthetic fungicides based on Prochloraz and benomyl completely inhibited the development of *Colletotrichum gloeosporioides* at higher concentrations. The EC_{50} and EC_{90} values are quite heterogeneous from one fungicide to another. Fongipro WG and Sphinx 480 WDG were the most effective on the basis of their lowest EC_{50} and EC_{90} against *Cercospora sp*. On the other hand, Nordox 75 WG was the least effective (its active ingredient, copper oxide, has a contact mode of action). This could be explain by the fact that, treatments started in the field when disease were already established. In fact, contact fungicides are suitable to apply for disease prevention than to disease eradication (Dekker, 2002).

Despite some constraints related to chemical control like toxicity and pollution, chemical fungicides remain one of the most effective and rapid means of controlling plant diseases. Cercospora leaf spot is a real pathological problem for seedling production of *E. saligna* and *P. africana* in Cameroon. Disease incidence and severity were very high in control seedlings of both tree species compared to seedlings treated with fungicides. This suggests that active ingredients of all the fungicides tested have antifungal properties against *Cercospora sp*, causal agent of Cercospora leaf spot. These results are similar to those obtained by Djeugap *et al.* (2015) who showed that chemical fungicides like Copper oxide and Metalaxyl inhibited the development of *Lasiodiplodia theobromae*, responsible of shoot blight of *Ricinodendron heudelotii*

Table 3: Effect of fungicides application on the inhibition (%)* of the growth of *Cercospora* sp.

Concentration	Fongipro WG	Fongistar 72 WG	Sphinx 480 WDG	Nordox 75 WG
Control	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
0.5 mg/ml	50.59±2.56b	35.25±3.25c	63.92±6.79a	32.16±5.56c
1 mg/ml	79.76±4.17a	57.25±4.01b	87.06±7.35a	39.22±2.96c
2 mg/ml	94.12±5.13a	85.88±6.73b	100.00±0.00a	64.31±4.90c

* Means in the line followed by different letters are significantly different according to the Duncan test at $P \leq 0.05$.

Table 4: Values of equivalent concentration (mg/ml)* for the inhibition of 50% (EC_{50}) and 90% (EC_{90}) of the radial growth of *Cercospora* sp.

Fongicide	Fongipro WG	Fongistar 72% WP	Sphinx 480 WDG	Nordox 75 WG
EC_{50}	0.53±0.2c	0.73±0.3b	0.49±0.2c	1.19±0.4a
EC_{90}	1.65±0.5c	2.61±0.7b	0.83±0.3d	5.07±1.2a

* Means in the line followed by different letters are significantly different according to the Duncan test at $P \leq 0.05$.

Table 5: Incidence* and severity* of Cercospora leaf spot of *Eucalyptus saligna* and *Prunus africana* seedlings after fungicides application.

Treatment	*E. saligna*		*P. africana*	
	Incidence	Severity	Incidence	Severity
Control	78.7±9.2a	54.3±8.2a	92.4±11.6a	66.3±6.6a
Fongipro WG	39.2±7.2b	27.3±5.1c	39.5±4.3b	31.4±4.6c
Fongistar 72% WP	41.5±8.1b	22.2±3.9c	47.1±5.2b	25.7±3.3c
Sphinx 480 WDG	44.1±8.9b	23.5±4.2c	43.2±6.1b	28.9±4.2c
Nordox 75 WG	67.6±9.5ab	48.5±7.5a	80.2±9.5a	49.1±5.8b

*Means in the column followed by different letters are significantly different according to the Duncan test at $P \leq 0.05$.

seedlings. Also, Serghat *et al.* (2004) and Vawdrey and Grice (2005), have shown that synthetic fungicides applied to seedlings prevent the development of fungi such as: *Helminthosporium oryzae*, *Pirucularia grisea*, *Mycosphaerella fijiensis* and *Phytophthora infestans*. The efficiency of fungicide formulations such as Fongistar, Fongipro WG and Sphinx 480 WDG which contain Metalaxyl, Chlorothalonil and Prothioconazole respectively could be due to the systemic mode of action of their active ingredients and their target in the fungal cell. In fact, Metalaxyl penetrates fungal cells and selectively interferes with DNA synthesis, inhibiting the growth of the mycelium and the formation of spores and haustoria (Wollgiehn *et al*., 1984). Chlorothalonil is an electrophile that inhibits thiol enzymes important for fungal spore germination and sulfhydryl groups important in glycolysis and fungal respiration (O'Malley, 2010). This fungicide also inhibits the formation of critical fungal cell membrane ergosterols and enzymatic reactions, ultimately leading to cell death (Dekker, 2002). Prothioconazole inhibits the biosynthesis of sterols and interfere with the synthesis of ergosterol in fungi, leading to morphological and functional changes in fungal cell membrane (Dekker, 2002). However, it should be noted that the effectiveness of a fungicide in disease control depends on many other factors, including the mobility of the active ingredient in the plant tissues, the host phenology, the biology of the pathogen and the climatic factors (Semal, 1989; Rapilly, 1991). It is, therefore, advisable to use fungicide formulation with systemic mode of action (penetrates and kill the pathogen inside plant tissues) than fungicide with contact mode of action in disease control when plants are already infected.

Conclusion

Seedlings of *E. saligna* and *P. africana* are colonized by several fungal species, the most common of which is *Cercospora sp*. In the study area, seedlings of *P. africana* were more susceptible to Cercospora leaf spot than seedlings of *E. saligna*. All synthetic fungicides tested exhibited an *in vitro* antifungal activity against *Cercospora* sp. Due to their *in vitro* and *in vivo* efficiency, fungicide formulations Fongipro WG, Fongistar 72% WP and Sphinx 480 WDG could be homologated for control of Cercospora leaf spot of *E. saligna* and *P. africana* seedlings. It was envisaged to complete the identification of fungal species associated with foliar diseases of seedlings of *E. saligna* and *P. africana* by using molecular tools.

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