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Disease resistant tree legumes for Agro-forestry in dry area.

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ABSTRACT

For any plantation programme in agro-forestry, the selected plants should be assessed first for their susceptibility to the pathogens prevalent in the locality of plantation. In the present study, seed and leaf infecting fungi of four tree legumes (*Acacia nilotica* Willd., *Butea monosperma* Roxb., *Delonix regia* (Hook.) Raf. and *Tamarindus indica* Linn.) of the family Leguminosae (Fabaceae) were isolated. Koch postulation studies confirm the presence of *Penicillium sp*. and *Rhizopus sp*. in seed infection, and in leaf infection mixed populations of fungi were observed. Effect of fungicides on isolated fungal pathogens and free phenol contents of investigated plants under control and inoculated conditions also observed. It was found that *A.nilotica* and *D. regia* are possibly more resistant to diseases at seedling stage.

Keywords: Fungicides, Koch postulation, Phenol, plant pathogen, tree legume.

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INTRODUCTION

In nature, plants growing in fields are exposed to several pathogenic organisms that cause diseases. Development of disease may be considered one of the biotic stresses that can affect plants normal growth and development and in extreme cases can kill the host plant. Fungi are an important group of organisms that are pathogenic to plants. Infection of the host plant by the causal organism may occur at different stages of development and also different parts of a plant. The fungi normally attack seeds during storage when the seed moisture content is in equilibrium with relative humidity greater than 65% [1]. Seeds are susceptible to microorganisms and lose their viability under tropical conditions [2]. In poor storage conditions seeds were found to carry various microorganisms, adversely affecting seed quality and viability [3,4]. Some plant pathologist [5-7] reported several diseases of seeds and seedlings of *Acacia* species. A number of mycoflora like *Rhizopus*, *Erysiphae*, *Verticillum*, *Penicillium* and *Thermomyces* were associated with tree seeds such as *Acacia*, *Albizia* and *Cassia* causing germination failure of such species [8].

Disease occurrence varies according to the climatic conditions under which the plant was grown. Seed treatments with antipathogenic agents and fungicides have proved useful in storing seeds against post-damage and loss of viability. Some scientist [9,10] isolated some seed borne fungi of forest tree like *Acacia*, *Albizia*, *Bauhinia* and studied their control using fungicides like Bavistin, Blitox and Dithane M-45. A number of workers [11-13] have tried to find out the suitable control measure for several fungal species under in vivo and in vitro conditions. Although plants lack an immune system, they are often found to be surprisingly resistant to diseases caused by Fungi. Plants produce a large, diverse array of organic compounds, like phenolics, alkaloids and other secondary metabolites that appear to have no direct function in growth and development, but serve as defense compounds against herbivores and pathogen [14]. It is an established fact that many secondary metabolites of plants, particularly phenolics impart a great role in conferring resistance in plants against different pathogens [15,16].

Before undertaking afforestation programme pathological study of the selected plants is essential, since incidence of pathogen frequently occurs through seeds as well as seedlings. In the present investigation with local trees, plants were first screened under field condition for occurrence of pathological diseases and the responsible organisms. Disease occurrence and causal organism was confirmed through Koch's postulation experiment. Next, pathogenic fungi were tested against fungicides for their sensitivity. Finally, seedlings of the selected plants were analyses for accumulation of phenolic substances as defense compound in response to pathogenic infection to specific fungi. This will give an insight of relative resistance of these fuel wood plants against fungal diseases.

MATERIALS AND METHODS

In the present investigation, four tree species (*Acacia nilotica* Willd., *Butea monosperma* Roxb., *Delonix regia* (Hook.) Raf. and *Tamarindus indica* Linn.) of the family Leguminosae (Fabaceae) were used and seedlings (20 days old) were taken as experimental plant materials to assess responses to pathogenic attack. All the plant samples in this research were taken from Bankura district which is the western most district of west Bengal. Bankura lies between 22° 38 ' and 23° 38 ' North latitude and between 86° 36 ' and 87° 46 ' East longitudes. The soil is mainly dry lateritic type and having the pH ranges from 5.5 to 6.5 at different regions of the district.

(A) Identification of plant pathogens responsible for diseases:

Seeds, leaves and stem lesions with infections were collected from in and around Bankura, surface sterilized with 0.1% H_gCl_2 for 2-3 minutes [17], washed three times with sterile water and dried with sterilized blotting paper before plating on to malt-agar medium. Infected portions were cut into small pieces and 3-4 such pieces were inoculated on ME-plate. The inoculated petridishes were incubated at 28 ± 2°C in BOD incubator for 3-5 days. The incubated dishes were examined for occurrence of any fungal or bacterial colonies. The colonies appeared on these plates were counted and characterized. One set of fungi appeared after incubation was preserved on malt-agar slants for further studies.

Staining procedure:

For general microscopic observation of the isolates, lactophenol cotton blue stain was used for staining. A few mycelia portion of each isolate was taken separately on clean slides. A few drops of lactophenol cotton blue were added on the mycelia. The preparation was fixed by gentle heat and the stain was allowed to react for 2-3 minutes at room temperature, washed thoroughly with lactophenol and finally mounted in lactophenol after proper teasing and examined under microscope. The nature of hyphae, reproductive structure and resting spores were noted.

Identification of the fungal culture was done by microscopic observation of the stained cultures following the standard literature [18,19].

(B) Confirmation of causal agent for diseases in seeds or seedlings by reinoculation (Koch's postulation):

For pathological study healthy seeds of four tree species Viz., *Acacia nilotica, Butea monosperma*, *Delonix regia* and *Tamarindus indica* were taken as plant material. After germination they were placed on sand beds to generate seedlings. Then 20 days old seedlings were taken as plant samples. Koch postulation experiments were performed using cultures isolated from the infected plant parts. This was done using conidia of freshly grown isolates against seedlings of their specific hosts (the plant species from which they were originally isolated). For this purpose, 20 days old seedlings were taken.

To infect, seedlings were first rubbed mildly with sterilized sand and then applied with conidia at different inoculums density (low [100 conidia ml^{-1}], medium [600-1200 conidia ml^{-1}] and high [3000 or more conidia ml^{-1}]) of all the fungal isolates. One control set was maintained. Five seedlings were inoculated for each treatment. After 15 days of treatment seedlings were observed carefully for the development of any symptoms comparable to their mother host. Very mild symptoms appeared on the leaves and stem treated with high inoculums density. Next, infected seedlings (leaves and stem) were cut into pieces, thoroughly surface sterilized with 0.1% H_gCl_2 for 2-3 minutes, washed three times with sterile water and dried with sterilized blotting paper before plating on to malt-agar medium (with same composition). The inoculated petridishes were incubated at 28 ± 2˚C in BOD incubator for 3-5 days. Seeds were also mixed with isolated pathogens similarly under aseptic condition. After 15 days only monocultures were obtained in most cases.

(C) Effect of fungicides on isolated fungal pathogens:

The fungi, which were isolated in earlier step, were then tested for their growth responses in presence of two common systemic fungicides viz., Blitox and Dithane M 45. Different concentrations (50-1000 µg ml^{-1}) of these fungicides were mixed with malt-agar medium and each fungus was tested for their ability for growth in presence of fungicides. The inoculated petridishes were incubated at 28 ± 2˚C in BOD incubator for 3-5 days.

(D) Assessment of defense against pathogens:

Assessment of the plant defense against pathogen was done by analyzing the accumulation of free phenols by the seedlings infected with specific pathogens. Seedlings of 20 days were first rubbed mildly with sterilized sand and then applied with conidia of pathogens producing leaf diseases (leaf curl and spot) at medium inoculums density. One control set was also maintained. Five seedlings were inoculated for each species. After 7 days of treatment leaves of the seedlings were analyzed for total free phenol content.

The extraction and estimation of the total free phenol content was done according to the method of [20]. The leaf samples (50 mg) were homogenized in 5 ml 80% ethanol and kept at 65^{8} C in a water bath for 5 minutes. The mixture was centrifuged at 5000 rpm for 10 minutes. The supernatant was taken in a beaker, 4 ml of distilled water was added and the mixture was kept on a hot water bath. After complete evaporation of alcohol, the aqueous phase was mixed with 4 ml of ether in a separating funnel and shaken thoroughly for 10 minutes. After a rest for a while the upper ether layer was taken and mixed with 3 ml hot distilled water and kept in open air until the ether was evaporated. One ml of this aqueous extract was taken and to this 3 ml of 5% Na_2CO_3 (w/v) and 1 ml of Folin phenol reagent were added and the mixture was kept in a boiling water bath for 1 minutes. Absorbance of the blue colour developed was measured at 650 nm. Content of phenol was

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calculated by comparing the absorbance with a standard curve prepared from catechol (Sigma Chemical Co., USA). The content of free phenol was expressed as mg g^{-1} fresh weight.

RESULT AND DISCUSSION

Infected seeds and leaves of the selected plants produced different fungal colonies, in ME plates, which are enlisted in Table 1. In infected seeds of *Acacia nilotica*, fungal species like *Verticillium* and *Erysiphae* were found. For leaf curl and leaf spot diseases, infected leaf produced colonies of *Phytophthora* sp. In case of *Butea monosperma*, seeds were infected with *Penicillium* sp. While *Erysiphae* sp. and *Taphrina* sp. were found to be associated with leaf curl diseases and other two fungal species like *Alternaria* sp. and *Curvularia* sp. responsible for leaf spot diseases. In case of *Delonix regia,* seed infection was due to *Penicillium* sp. and leaf curl diseases was due to *Phytophthora* sp. and one other unknown fungal species. *Tamarindus indica* seed were infected by *Thermomyces* sp. and *Penicillium* sp. while in case of leaf spot disease the fungal colonies produced on culture medium was very much similar to *Curvularia* sp. and *Alternaria* sp.

Table 1: Fungal pathogens causing diseases of four tree legumes

Plant Species	Infected Plant Parts	Name of the Pathogens
Acacia nilotica	Seed	*Verticillium* sp., *Erysiphae* sp.
	Leaf curl	*Phytophthora* sp., *Curvularia* sp.
	Leaf Spot	*Phytophthora* sp.
Butea monosperma	Seed	*Penicillium* sp.
	Leaf curl	*Erysiphae* sp., *Taphrina* sp.
	Leaf spot	*Alternaria* sp., *Curvularia* sp.
Delonix regia	Seed	*Penicillium* sp.
	Leaf spot	*Phytophthora* sp., *Curvularia* sp.
Tamarindus indica	Seed	*Thermomyces* sp., *Penicillium* sp.
	Leaf spot	*Curvularia* sp., *Alternaria* sp.

Table 2: Organisms isolated after Koch's postulation experiments.

Plant Species	Seed infection	Leaf spot	Leaf curl
Acacia nilotica	*Verticillium* sp.	*Phytophthora* sp.	*Phytophthora* sp.
Butea monosperma	*Penicillium* sp.	*Alternaria* sp.	*Erysiphae* sp.
Delonix regia	*Penicillium* sp.	*Phytophthora* sp.	-
Tamarindus indica	*Penicillium* sp.	*Curvularia* sp.	-

Table 3: Fungicide tolerance level of the isolated pathogens (after Koch's postulation against Blitox and Dithane M-45)

	Blitox (µg ml^{-1})						Dithane M 45 (µg ml^{-1})					
	0	50	100	200	500	1000	0	50	100	200	500	1000
Seed												
Penicillium sp.	+	+	+	+	+	-	+	+	+	+	-	-
Verticillium sp.	+	+	+	+	+	-	+	+	+	+		-
Leaf Curl/Spot												
Curvularia sp.	+	+	+	+	-	-	+	+	+	+	-	-
Phytophthora sp.	+	+	+	-	-	-	+	+	+	+	-	-
Alternaria sp.	+	+	+	+	-	-	+	+	+	-	-	-
Erysiphae sp.	+	+	+	+	-	-	+	+	+	+	-	-

After re-inoculation of the pathogens (fresh conidia) in seed or young seedlings (20 days old) of the respective plants followed by inoculation into malt agar medium, fungal colonies produced in ME – plates were found to be less in number compared to earlier (Table 2) for each plant species. Thus only *Verticillium* species was observed in case of seed infection of *A .nilotica* after Koch's postulation. While for leaf infection of

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young seedlings of this species single colony of *Phytophthora* sp. could be isolated. After Koch's postulation experiment, infected seeds of *B. monosperma* showed only *Penicillium* species thus confirming this fungus as the casual organism of seed infection. Similarly, *Alternaria* sp. and *Erysiphae* sp. caused leaf spot and leaf curl diseases, respectively. Newly infected seeds of *D. regia* produced colonies of *Penicillium* sp. and infected leaf part showed the presence of *Phytophthora* sp. in ME medium. In case of seed of *T. indica* monoculture of *Penicillium* sp. was isolated, while infected leaves produced colonies of *Curvularia* sp. only. Here also *Alternaria* and some other unknown fungi, which are found earlier, were totally absent in reisolated plates.

Chemicals are commonly, successfully and economically used to control seed and seedling diseases of tree legumes [11,13]. The fungi, which were re-isolated, were then tested for their sensitivity to two common systemic fungicides (Blitox and Dithane M -45). It was observed that (Table 3) the growth of the fungi responsible for seed infection (Viz., species of *Penicillium* and *Verticillium*) was not affected by lower concentration of both the fungicides (50 – 500 µg ml $^{-1}$), but at higher concentration (1000 µg ml $^{-1}$) colonies of those organisms were not formed at all in ME culture medium. In case of fungal species causing leaf diseases (either leaf spot or leaf curl) in all the tree legumes, 200 µg ml $^{-1}$ concentration and above inhibited growth as no colony appeared in these concentrations.

When seedlings (20 days old) of the plants were inoculated with pathogens responsible for leaf diseases phenol content increased in leaves of all species (Fig. 1). However, such increase was highest in *A. nilotica* seedlings followed by *D. regia*, while *B. monosperma* seedlings showed lowest amount of phenol among all species.

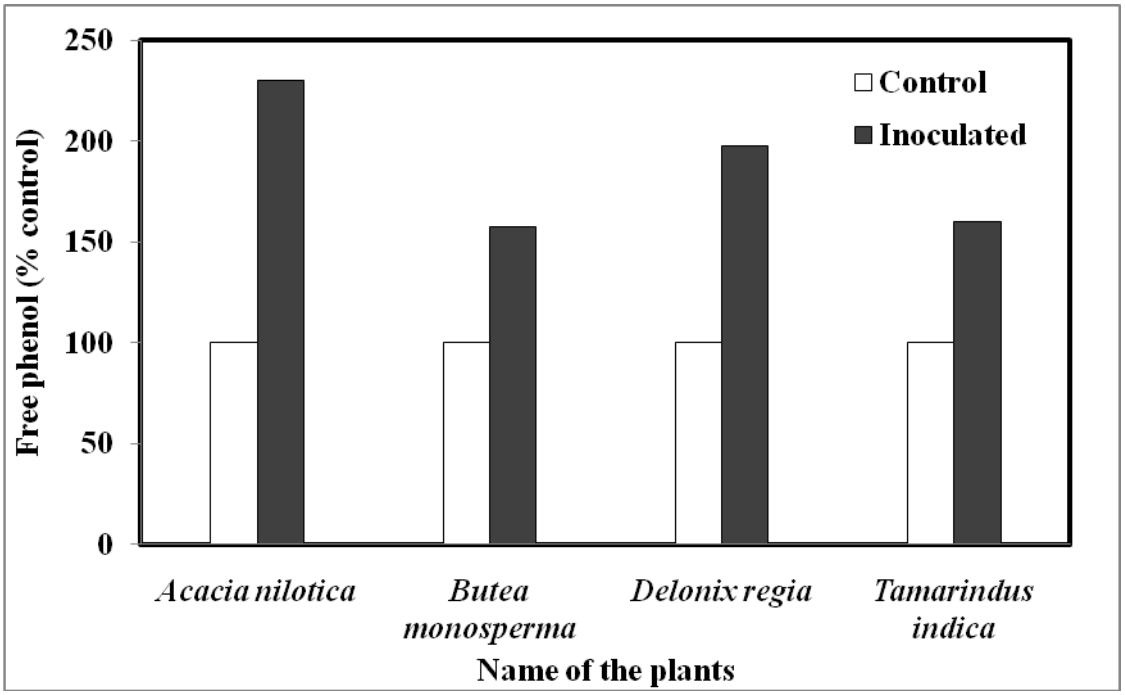

Fig 1: Free phenol contents of investigated plants under inoculated condition (by pathogen responsible for leaf infection).

For any plantation programme, the selected plants should be assessed first for their susceptibility to the pathogens prevalent in the locality of plantation. In afforestation programme, large quantities of seeds of forest trees are being collected and used for raising seedlings in nurseries. Depending on moisture content seed can carry a number of pathogenic organisms and unhealthy seeds have the potential of introducing dangerous diseases to new plantation areas. Mostly fungi as compared to bacteria, viruses or nematodes cause large number of plant diseases that occur more commonly in seeds.

Several workers reported about different types of fungi responsible for seed and seedling diseases. In the present investigation, causal organism of seed infection was *Penicillium* (for *B. monosperma*, *D. regia* and *T. indica*) and *Verticillium* species (for *A. nilotica*) and the causal organism of leaf curl was *Phytophthora* (for *A.*

nilotica) and *Erysiphae* species (for *B. monosperma*). For leaf spot infection, the causal organism was confirmed to be *Phytophthora* species for both *A. nilotica* and *D. regia*. While *Alternaria* and *Curvularia* species were confirmed to be the causal organisms for the leaf spot diseases of *B. monosperma* and *T. indica* respectively.

In the present study, it was observed that the fungi responsible for seed infection of these trees viz., *Penicillium* and *Verticillium* species were found to be less sensitive since their growth was inhibited only at higher concentration (1000 µg ml $^{-1}$) in ME medium. On the other hand, fungi responsible for leaf diseases viz., *Alternaria*, *Curvularia*, *Phytophthora* and *Erysiphae* species were found to be far more sensitive to fungicides as they can be controlled at 200 µg ml $^{-1}$ concentration for both fungicides. There are variable reports on the effectiveness of the fungicides to control fungal diseases. In the present study, both the fungicides affected the growth of the test fungi (responsible for leaf diseases) remarkably at 200 µg ml $^{-1}$concentration. Limited work has been done on control of foliage diseases of tree legumes. Tree pathologists have generally minimized the importance of foliar diseases as they rarely affect timber production, usually the most valuable forest product. However, if the tree legume is grown as a source of forage and green manure, then the effect of foliar diseases is very important.

When seedlings were inoculated with the pathogens responsible for specific leaf diseases, phenol content increased in leaves of all species. It is an established fact that many secondary metabolites of plants particularly phenolics impart a great role in conferring inducible resistance to the plant against different pathogens. However, such increase was highest in *A. nilotica* seedlings followed by *D. regia*. This intrinsic character of the plant may be inducible in nature, which helps plants to overcome the unfavorable condition. Thus *A. nilotica* and *D. regia* are possibly more resistant to diseases at seedling stage.

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