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Growth Response of Uapaca. Kirkiana Seedlings to Ectomycorrhizal Inocculation in Sand Growth Media

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Abstract. Symbiotic efficiency of mycorrhizal fungi on the establishment and growth of *Uapaca.kirkina* seedlings was studied by use of *Amanita zambiana, Cantharellus cibarius, Cantharellus* sp., *Lactarius deliciosus,* and *Lactarius edulis*. Five grams of the respective fungal inoculum were used in each polythene pots. Treatments were replicated ten times and arranged in a completely randomised design. Seedlings raised in sterilised pine bark were transplanted into the inoculated potted soil and control polythene pots. Growth response of the seedlings was assessed at monthly interval.. Seedlings inoculated with an autoclaved mycorrhizal fungi mixture inoculum, had 100% survival rate compared to the seedlings inoculated with the live mixture of mycorrhizal fungi inoculum which had 100% mortality. L. deliciosus inoculated seedlings had the highest growth response followed by the control-1.The paper discusses soil myco-ecological factors that could possibly have influenced seedlings growth response variation.

Keywords: Uapaca kirkiana, mycorrhizae, fungi, sporocarp, inoculums.

1. Introduction

The study on the ecology and pattern of mycorrhizal fungi distribution in the *Uapaca kirkiana* woodlands [1] indicated the presence of particular fungi populations, but it did not qualitatively or quantitatively exhibit their ecological efficiency and compatibility with *U. kirkiana.* The quantitative assessment of the efficiency of mycorrhizal fungi involves determining the ability and extent individual fungi enhance nutrient and water uptake by plants, lengthen life of host tree roots, protect the host tree root systems against soil-borne pathogens, and also their ability to increase tolerance of plant roots to various adverse soil conditions [2]-[3]. The ecological complexities of the mycorrhizal fungi occurring in nature, nevertheless, make controlled laboratory experiments evaluating the above individual fungal functions of limited practical benefit. It is the cumulative effect and interactions of these factors rather than their individual effect that influence plant biomass production or seedling growth. As mycorrhizal fungi are known to be important for seedling establishment because of their wide range of functions [4], the most practical way of assessing their efficiency is by determining plant growth response to their influence. Several mycorrhizal fungi plant growth response studies have been carried out elsewhere for different tree species [5]-[9].

Variations in symbiotic efficiency of mycorrhizal fungi have been proved in various experiments. [10] working on *Larix* kaemferi established that five fungal species had varying effects on seedling growth assessed by root and shoot length. Similarly, work by Singh and Lakhanpal [11] studying mycorrhizal fungi *Boletus edulis* and *Russula brevipes* revealed variation in their effect on seedling biomass and root collar diameter. Related studies on different tree species and mycorrhizal fungi have been carried out elsewhere [12]-[17].

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Differences in the influence of mycorrhizal fungi species on the growth of host plants could be due to variation in their percentage root infection. It has also been established in pure culture experiments that mycorrhizal fungi also differ in their ability to use various carbon and nitrogen compounds, growth substances requirements, and their relation to pH, temperature, and oxygen supply [18]-[21]. Results from the plant growth response experiments by various workers have however, tended to be inconsistent. This could be due to several factors, such as the influence of different artificial laboratory or green house nursery conditions. Spores of certain fungal species fail to germinate under certain nursery conditions. In addition, fungal mycelial growth and infection processes are generally highly influenced by aeration, soil structure and many other factors such as pH, soil nutrient levels, and temperature [22]-[24]. Given that *U. kirkiana* seedlings have proved difficult to raise under nursery conditions, this research was undertaken to establish from the identified mycorrhizal fungi the most efficient species in enhancing seedling survival and growth under such conditions.

The specific objectives of the study were the following:

(i) Assess influence of mycorrhizal fungi on seedling growth.

(ii) Evaluate colonisation efficiency of the selected mycorrhizal fungi species.

2. Methods and Materials

2.1. Identification of study sites

Ten study sites were identified by use of vegetation map and field visits along the *U. kirkiana* dominated miombo woodland belt occurring in the Zimbabwe highveld. Selection of sites was undertaken in such a way that a wide range of varying rainfall, soil and vegetation conditions was represented. Changes in the state of the vegetation in most cases were noted to have been highly influenced by human activities and this was also taken into consideration when these study sites were selected.

2.2. Preparation of mycorrhizal fungi inoculum

Five mycorrhizal fungi that were commonly found in the ten study sites were chosen for this experiment, i.e. *Amanita zambiana, Cantharellus cibarius, Cantharellus* sp., *Lactarius deliciosus,* and *Lactarius edulis.* Sporocarps of these fungi were collected from *U. kirkiana* woodland and air-dried in khaki pockets. The respective dried sporocarps were ground with a laboratory grinder and sieved through a 2 mm mesh to produce a fine inoculum powder. Twenty grams of each of the fungi spore collections was used for nutrient analysis using spectrophotometry [25]. The remaining powder was kept in vials at 4^0C in an incubator [26] for one week and used as inocula for the experiment.

2.3. Mycorrhizal fungi nutrient composition analysis

To understand the nutrient transport system from the soil across to the roots of host plants, sporocarps of selected species of the common edible mycorrhizal fungi species were analysed for their nutrient composition. Nutrient levels of the respective mycorrhizal fungi could be indicative of the fungi being either a sequester or efficient nutrient transporter. P content was analysed using the method described by Koroleff [25], N was analysed using the Kjeldahl method and the exchangeable bases were analysed by the method described by Hesse [27]).

2.4. Inoculation treatments

Eight inoculation treatments were formulated as follows: (i) *Amanita zambiana (*T1*),* (ii) *Cantharellus cibarius* (T2), (iii) *Cantharellus* sp (T3)., (iv) *Lactarius edulis* (T4), (v) *Lactarius deliciosus* (T5), (vi) Cocktail (mixture) of the five mycorrhizal fungi species-(T6), (vii) Control-1 (autoclaved cocktail of mycorrhizal fungi)-T7, (viii) Control–2 (uninoculated)-T8.

Five grams of the respective inoculum were placed at 4 cm depth of the sterilised sand media in polythene pots that had a diameter of 5 cm and depth of 12 cm. These eight treatments were replicated ten times and arranged in a completely randomised block. *U. kirkiana* seedlings raised in sterilised pine bark were transplanted into the inoculated and control polythene pots when the first pair of leaves had developed. One seedling was planted per pot making a total of 10 plants per replication per treatment. These seedlings

were raised in a glass-house that had day temperature that varied between 25^0C and 36^0C. Seedlings were fertilised by weekly application of liquid fertiliser (75 ml per pot) which had the following nutrient composition; 5N, $6P_2O_5$, $7K_2O_5$, Mg (0.1%), Zn (0.03%), Cu (0.02%), B (0.03%) and S (0.15%). Watering was carried out when necessary to meet their moisture requirements. All seedlings received full sunlight for an 8-hour photoperiod per day. As the growth media had been sterilised, few weeds occurred and were removed by hand. Seedling growth assessments were carried out on a monthly basis for a period of four months. Growth parameters that were assessed were: shoot height, leaf number, total fresh and dry mass.

Macroscopic features were used to examine fine roots to determine presence or absence of mycorrhizal colonisation when the seedlings were harvested after four months. The presence or absence of hyphae and rhizomorphs and the branching habit of fine roots were noted. Sub-samples of fine roots of each treatment were examined, using the trypan blue stain method and percentage infection estimated by use of stereo-microscope to determine the ratio of 'number of mycorrhizal short roots: total number of short roots examined'. Final shoot heights were measured at harvest. In addition, root and shoot mass were recorded after drying to a constant mass at 70^0C. Analysis of variance was used to identify significant responses.

As defoliation, die-back and seedling mortality were also considered as effects of the various treatments, missing values were not extrapolated, but only complete data sets were analysed. Forty observations for the leaf number assessment, 57 observations for seedling biomass assessment and 55 observations for mycorrhizal root colonisation were analysed. Following the F-test, where significance was detected, Tukey's test was used to separate the means at the 5% level of significance.

3. Results

3.1. Mycorrhizal fungi nutrient composition

Generally all the mycorrhizal fungi showed to be accumulator fungal species, sequestering the following micro elements: Fe, Mn, Zn, B and Cu. *L. deliciosus* was noted to be superior in the sequestration of all the elements and Fe being the highest at 1238 mg/kg. Rate of sequestration of minerals is not however indicative of the symbiotic efficiency of the mycorrhizal fungi. Results of the mycorrhizal fungi nutrient analysis are shown in Table. 1.

Table 1: Nutrient composition of the common mycorrhizal fungi

Mycorrhizal fungi	Macro-elements (%)					Micro-elements (mg/kg)				
	N	P	K	Ca	Mg	Fe	Mn	Zn	B	Cu
C. cibarius	3.22	0.62	3.60	0.23	0.13	555	50	89	12	97
L. deliciosus	4.92	0.72	2.24	0.23	0.11	1238	62	134	12	102
L. edulis	3.18	0.62	3.61	0.20	0.13	668	37	90	12	98
A. zambiana	4.44	0.84	4.65	0.25	0.13	184	20	100	9	24
Cantharellus sp.	3.22	0.44	3.43	0.23	0.10	483	20	64	12	50

3.2. Seedling growth assessment

One month after transplanting, most seedlings had three leaves above the premordial leaves. At the second assessment, seedlings inoculated with a cocktail of mycorrhizal fungi were showing symptoms of drying and all those seedlings had died by the time of the third assessment. The analysis of variance for the effect of various treatments on survival and the growth factors indicate that there were statistically significant differences in the percentage seedling survival ($P<0.01$) and in the number of leaves ($P<0.05$) between treatments but no differences in shoot length were noted .Data on leaf number, fungi colonisation percentage and seedling total dry mass from the eight treatments were analysed (one-way analysis) with SAS statistical software using the following model:

$$Yij = \mu + \alpha i + \varepsilon ij$$

Where: Yij =jth observation in the ith treatment

μ = overall mean

αi = effect of ith treatment, i = 1,2,3,…,8

εij = random error component

Seedlings inoculated with an inoculum of autoclaved mycorrhizal fungi (mixture), had 100% survival rate whereas the seedlings inoculated with the live inoculum mixture of mycorrhizal fungi had 100% mortality. *L. deliciosus* inoculated seedlings had the highest number of leaves compared to the other treatments followed by the control-1 (autoclaved mycorrhizal fungi cocktail). The superiority of influence of *L deliciosus* on the number of leaves was also noted to have a corresponding positive inflluence on the seedling height. Seedlings inoculated with *L deliciosus* attained mean height of 7.40 cm followed by seedlings incoulated with *A.zambiana* which attained a seedling mean height of 7.33 cm.

3.3. Percent root colonisation and biomass production

There were statistically significant differences between treatments in percentage root colonisation by mycorrhizal fungi ($P<0.05$). *A. zambiana* had the highest percentage colonisation of over 60%, followed by *Cantharellus* sp. which had 52%, and the rest had values of 40% and lower (Table 2).

Table 2: Root colonisation percentages of different mycorrhizal fungi*

Treatment	Mycorrhizal fungi species				
	C. cibarius	*L. edulis*	*L. deliciosus*	*A. zambiana*	*Cantharellus* sp.
code#	T1	T2	T3	T4	T5
% Colonisation	40.17bc	40.11bc	34.43bc	64.19a	52.10ab

* Values within the row with the same letter are not statistically significantly different at 5%. Tukey's Studentised Range Test was used to compare the means.

Effects of different treatments on the seedling biomass production were also analysed using ANOVA. The results indicated that the total dry mass of the different treatments were statistically significantly different at $P<0.01$. With respect to biomass production, seedlings inoculated with the autoclaved mycorrhizal fungi cocktail had the highest total dry mass followed by those inoculated with *A. zambiana*, while the control-2 (uninoculated) had the least total dry mass (Table 3).

Table 3: Seedling biomass production for different mycorrhizal fungi treatments at 4-months stage of growth

Treatment (Inoculum)	Code	Mean total dry mass* (g)	Mean dry shoot mass (g)	Mean dry root mass (g)	Shoot-Root ratio
Cantharellus cibarius	T1	0.36bc	0.29bc	0.07bc	4.10
Lactarius edulis	T2	0.30c	0.24b	0.06b	4.00
Lactarius deliciosus	T3	0.30c	0.25b	0.05b	5.00
Amanita zambiana	T4	0.47b	0.36c	0.12c	3.00
Cantharellus sp.	T5	0.38bc	0.30b	0.08b	3.73
Mycorrhizal fungi cocktail #	-	-	-	-	-

The good growth of seedlings inoculated with autoclaved inoculum was probably because of beneficial effects of additional micronutrients found in the fungi combination (see Table 1 for nutrient composition of different fungal species). In assessing the influence of individual mycorrhizal fungi on biomass production, it is evident that mycorrhizal root percentage colonisation is an important factor. For example, *A. zambiana* had both the highest fungi colonisation (Table 2) and the highest mean biomass (Table 3). Such results tend to suggest that under certain conditions, seedlings inoculated with selected mycorrhizal fungi species could perform better than the uninoculated ones.

4. Discussion

In the comparative analysis of influence of the various mycorrhizal fungi on the *U. kirkiana* seedling growth, there were statistically significant differences ($P<0.05$) between different treatments. The improved growth of seedlings inoculated with autoclaved inoculum that accumulated the highest mean total dry weight of 0.76 g was probably due to the beneficial effect of additional combined effects of micro-nutrients found in the inoculum (see Table 1 for nutrient composition of different fungi). This implies that the mycorrhizal fungi used in this treatment are 'accumulator species', sequestering more nutrients than they supply to the host plant. Iron, the element not supplied by liquid fertiliser but found in all test fungi, has been noted to be an important plant element in miombo woodland ecosystem [28] Contribution of macronutrients found in the

autoclaved inoculum would mostly be insignificant when compared to the micronutrients, which are required in small quantities. Variation in the influence of individual mycorrhizal fungi could be due to inherent differences in the colonisation efficiency, mycelial growth rates and response to environmental factors.

Among the five mycorrhizal fungi inoculants, *L. deliciosus* exhibited the greatest influence on the seedling height, followed by *A. zambiana*. *L. edulis* had the least influence, probably reflecting its poor symbiotic efficiency. Symbiotic efficiency of mycorrhizal fungi has been suggested by Bowen [29] to be dependent on the combination of influence of environment on pre-infection phases such as spore germination and mycelial growth through the soil and the root susceptibility. Factors that can influence variation in basidiospore germination percentages between different fungi could be their respective differences in their responses to root exudates. Several workers have established that certain types of fungi spores germinate when they come in contact with root exudates, and the other group involve species whose spores are not stimulated by root exudates [30]-[32]. The first group has been termed 'early stage' fungi and the second group the 'late stage' fungi. It can therefore be assumed that when spores are used as inoculum, late stage fungi would not prolifically form mycorrhizae at an early stage. Fleming [33] suggested that infection by late stage fungi occur through mycelia. It is therefore important that investigations be carried out to establish whether organic material or microorganisms associated with organic material stimulate spore germination of late stage fungi, or whether exudates from older parts of roots stimulate them. The 'early stage' fungi have been reported to include species of the genera *Hebeloma*, *Laccaria* and *Pisolithus*, and the 'late stage' fungi include species of *Amanit*a, *Russula*, *Lactarius* and *Leccinum*. In this experiment, *Lactarius deliciosus* and *A. zambiana*, although belonging to the late stage fungi group, produced the best seedling growth responses when compared to other species, probably indicating that their spores managed to germinate and infect the seedling root systems. This highlights that the theory of 'early' and 'late stage' fungi still needs to be further researched. Furthermore, reduction of other beneficial micro-organisms through sterilisation could also have affected performance of certain fungi species. Mycorrhizal fungi function in collaboration with various other microbes. For instance, Duponnois [34] identified mycorrhizal helper bacteria that promoted ectomycorrhizal establishment of *Laccaria lacuna* but inhibited mycorrhizal formation by other fungi.

Influence of individual mycorrhizal fungi on the physiology of seedlings being measured by biomass accumulation, showed *A. zambiana* to be significantly superior ($P<0.05$) to L*actarius edulis*, *L. deliciosus* and control-2 (Table 3). When compared to other mycorrhizal fungi treatments, seedlings inoculated with *A. zambiana* had the lowest shoot-root ratio of 3, indicating an increase in root volume. This increase in root volume could be as a result of its relatively high root percentage colonisation of 64.19 %.

Although it has been shown that differences exist between different fungal species in their influence on the growth of *U. kirkiana* seedlings, it is recommended that the identified fungi be further evaluated under different environmental conditions. Work on various mycorrhizal fungi has been reported to have variable results under different conditions. For example, inoculation with *Paxillus involutus* greatly improved growth and survival of pine seedlings in the field [22], whereas in the experiment by Lundeberg [35], the same fungus suppressed seedling growth. Likewise the fungus *Cenococcum graniforme*, which is very drought tolerant and therefore has been recommended for dry areas [36], has inhibited growth of pine seedlings in some nursery experiments. The observed variation could most likely be explained by individual fungi species differences in their tolerance to various nursery conditions. Different fungi have different optimum environmental conditions and therefore cannot perform the same under artificial nursery conditions. It should therefore be noted that when screening fungi species in controlled artificial environments, it should not be assumed that they would perform the same in nature. On the other hand, it is also difficult to accurately study functions of the respective fungi in nature because of the dynamics of microbial populations in nature and their complex biochemical reactions that occur in the soil. The complexity of myco-ecology in natural systems makes it difficult to isolate and accurately study various factors and assess their effects on the performance of respective fungi species. It can therefore be concluded that laboratory or nursery comparative studies of individual mycorrhizal fungi efficiency would be unlikely to provide reliable information on their field functional capabilities, but it is still a useful technique for identifying potential species.

Despite the evident beneficial effect of individual mycorrhizal fungi, the high mortality (100%) experienced by seedlings inoculated with a mixture of mycorrhizal fungi was indicative that under certain

conditions mycorrhizal fungi can exert negative effects on the seedlings. Similar occurrence has been reported Dominik [37] who indicated that under adverse nursery conditions the dominance of *Boletus luteus* caused mortality of pine seedlings. There are other studies that have reported situations when profuse growth of mycorrhizal fungi resulted in suppressed growth [38], [9], [2] also highlighted that under certain conditions, mycorrhizal fungi could cause photosynthetic drain on the host plant. It was suggested that this photosynthetic drain on the host plants often happen when there is an imbalance in the symbiotic relationship [39], and it was also established in their studies that cotyledons are the sole source of carbon needed for early mycorrhizal colonisation in miombo tree seedlings such that when cotyledons of seedlings are shed too early, the mycorrhizal fungi would wholly depend on the host seedlings for their carbon requirements. This condition would therefore cause photosynthetic drain on the seedlings. Wright *et al.* [40] also carried out comparative analysis of carbon physiological processes between mycorrhizal and non-mycorrhizal seedlings of *Betula pachela* and demonstrated that seedlings colonised by *Paxillus involutus* had significantly reduced biomass after six months when compared with the uninoculated. Similar results in nursery studies on the inoculation of pine seedlings with *Pisolithus tinctorius* have attributed this suppressed seedling growth to the drain of photosynthate (sucrose) due to the mycorrhizal demand. This mycorrhizal demand on the host has been estimated to be 10% of the carbohydrates produced by the tree hosts [41]. Suppression of seedling growth could possibly be caused by disproportional allocation of more C to the roots for the support of ectomycorrhizae demand than to the shoots. Hö rgberg *et. al* [42] reported that under low soil fertility trees allocate more C to the root than shoots.

Although these conditions may exert negative effects on the seedlings, it is, nevertheless, most unlikely that they would have resulted in the total mortality of the *U. kirkiana* seedlings. It has been established that mycorrhizal fungi demand on the host plant can be considerable but the benefits from the association under natural conditions usually outweigh the energy cost by the fungus. The host plant may be able to compensate for an energy drain to the fungus by increasing its photosynthesis [43].

Various other arguments have been advanced to explain mortality of miombo tree species seedlings under nursery conditions. Munyanziza [44], working on miombo tree species, investigated the effects of fertilisation on *Pterocarpus angolensis* and also evaluated the effects of different watering regimes on *Afzelia quanzensis* seedlings.

Fertilisation studies established that the normal shoot: root ratio of miombo tree species is disrupted when commercial N and P fertilisers are used in the nursery. It was noted that whereas the shoot: root ratio of *Pterocarpus angolensis* seedlings growing in a natural ecosystem had ratios below 2, this ratio shifted to above 2 when fertilised with N and P. Disturbance of the shoot: root ratio in the miombo tree species through various nursery management techniques such as manipulating root and root environment is considered to act against the natural seedling surviving strategy [44]. In nature, the miombo tree species develop a strong taproot, which is allocated a larger share of photosynthetic biomass. The annual shoots die back for 8 to 10 years until sufficient food and energy reserves have accumulated in the root system [45]-[47]. It has been suggested that it is a mechanism by which seedlings develop food reserves in the root system for them to survive during stress periods. Shoot die-back of miombo tree seedlings in natural ecosystems has been known to be triggered by droughts, and other environmental stresses [48]-[50]. This die-back does not generally lead to the physiological death of seedlings. The taproot survives the adverse dry periods and new roots and shoots emerge when rain resumes. It serves as a reservoir of water and nutrients [45], [51]-[52] further highlighted that taproots have access to deeper water and nutrients via inter-hyphal connections with the root systems of larger trees. It can therefore be concluded that in this study, the root systems were confined to limited growing medium in the polythene pots and as such they would not receive a supplementary supply of photosynthetic products through the inter-mycelial root connection that exists in nature. Table 3 shows the shoot: root ratios for the various treatments ranged from 2.04 to 5, indicating that there was more shoot growth than the root development for all the treatments. It can therefore be concluded that use of liquid fertiliser with the following nutrient composition: 5%N, 6%P_2O_5, 7%K_2O_5, Mg (0.1%), Zn (0.03%), Cu (0.02%), B (0.03%) and S (0.15%), disrupted the shoot: root ratio in all the treatments. Nevertheless, it cannot be concluded that disruption of the shoot: root ratio caused mortality of the various treatments because there is no correlation between shoot: root ratio and the percent survival ($r^2 = 0.02$).

Watering regime studies on *Afzelia quanzensis* revealed that moisture stress affected the photosynthetic capacity of seedlings [44]. This indirectly affected the amount of carbon available for the fungal symbiont. Lack of this carbon would therefore affect mycorrhizal fungi colonisation processes. This has not been examined in this study.

As the cause of the seedling mortality in this experiment cannot be conclusively ascertained, further studies need to be carried out to examine the effect of various possible adverse nursery conditions. An area that requires further investigation is the effect of factors such as soil compaction, fertilisation, pH, and moisture stress on the association of mycorrhizal fungi with the host seedlings and their survival. Studies to establish the optimum inoculum level for various mycorrhizal fungi should also be undertaken.

The study has, nevertheless, established that different mycorrhizal fungi vary in their influence on seedling growth. From the mycorrhizal fungi species under study, *L. deliciosus* was noted to have the greatest influence on seedling height but less effective on the seedling biomass, where *A. zambiana* proved superior to the rest of the species. Furthermore, these mycorrhizal fungi have been found to vary in their root colonisation efficiency. *A. zambiana* had the highest colonisation percentage followed by *Cantharellus* sp. Mycorrhizal fungi *L. deliciosus* had the lowest colonisation percentage.

It is therefore recommended that when mycorrhizal fungi have been identified under laboratory and nursery conditions to have have potential to be used as inocula, they should be further evaluated under different site conditions, particularly in areas where the seedlings are to be established. It may also be important to examine the behaviour of introduced mycobionts after out-planting because different soils have been shown to vary in their receptivity to different mycorrhizal fungi [53]-[55], [21]. Although results in this experiment indicated that all the tested mycorrhizal fungi, i.e. *A. zambiana*, *Cantharellus cibarius*, *Cantharellus* sp., *L. edulis* and *L. deliciosus* did stimulate seedling growth, there is still need to understand ecological processes involved. Areas that require further attention include, understanding effectiveness of different types of inocula (mycelia, spores), and the quantitative study of mycelial growth into the soil of these mycorrhizal fungi and other selected species found in the *U. kirkiana* ecosystem.

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