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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing *Escherichia coli* in the Calgary Health Region: emergence of CTX-M-15-producing isolates. *Antimicrob. Agents Chemother.* 51: 1281-1286.

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### ARTICLES

- Morphological and molecular identification of *Pythium* spp. isolated from common beans (*Phaseolus vulgaris*) infected with root rot disease** 1  
Papias H. Binagwa, Conrad K. Bonsi, Susan N. Msolla and Innocent I. Ritte
- Effect of poultry manure treated and untreated with effective microorganisms on growth performance and insect pest infestation on *Amaranthus hybridus*** 10  
Abiodun Joseph, Benson Oluwafemi Ademiluyi, Patrick Ajibola Aluko and Temitayo Martha Alabeni
- Mistletoe presence on five tree species of Samaru area, Nigeria** 16  
Tizhe Tari Dlama, Alonge Samson Oluwagbemileke and Aliyu Ramatu Enehezeyi
- Rooting and establishment of *Limoniastrum monopetalum* (L.) Boiss stem-tip cuttings** 23  
Anastasia Akoumianaki-loannidou, Aekaterini N. Martini and Maria Papafotiou
- Effects of soil substrate quantity and sowing method on *Cocoa* (*Theobroma cacao* L.) seedlings growth in Togo** 32  
**AyiKoffi ADDEN, Gbénonchi MAWUSSI, Robert Madjoulba**  
BATOCFETOU, Timondjro KOUDJEGA, Komla SANDA and Kouami KOKOU
- Iphimeis dives* (Crysomelidae) Beetle occurrence in beans in western Parana State, Brazil** 39  
André Luiz Alves, Meirieli Nunes, Antonio Carlos Torres da Costa, José Barbosa Duarte Júnior and Vanda Pietrowski

## Full Length Research Paper

# Morphological and molecular identification of *Pythium* spp. isolated from common beans (*Phaseolus vulgaris*) infected with root rot disease

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Common beans (*Phaseolus vulgaris* L.) is the main leguminous crop grown primarily by small-holder farmers in the East and South African countries. *Pythium* root rot disease is the major production constraints which results in yield losses of 70% to most commercial bean cultivars in eastern Africa. Study focused on ascertaining preliminary information on bean cultivation practices in Tanzania, morphological and molecular characterization and identification of *Pythium* species from infected beans plants and determining the relationship between soil pH and the occurrence and distribution of the *Pythium* spp. Soil samples and infected bean plants were collected by aseptic pathogenic isolation and DNA extraction. Universal primers (ITS1 and ITS4) were used for amplification and followed by sequencing. About 63.0% of farmers practiced sole beans cropping, 31.0% mixed cropping and 6.0% intercropping. Corn, banana, cassava, Irish potatoes and coffee were either mixed or intercropped with beans. Also, 52.4% of farmers use farm saved seeds and 92.9% do not use fertilizer in their bean fields. Eleven species of the *Pythium* spp. were identified: *Pythium aphanidermatum*, *Pythium splendens*, *Pythium ultimum*, *Pythium attrantheridium*, *Pythium graminicola*, *Pythium oligandrum*, *Pythium dissotocum*, *Pythium irregurale*, *Pythium camurandrum*, *Pythium paroeandrum* and *Pythium acanthophoron*. Phylogenetic analysis showed diversity and homogeneity among the *Pythium* spp. across the collection area. A high incidence and wide distribution of *Pythium* species were recorded in soils in the 5.03 to 5.95 pH range.

**Key words:** Incidence, internal transcribed spacer (ITS), leguminous, molecular characterization of pathogen.

## INTRODUCTION

Common beans (*Phaseolus vulgaris* L.) is one of the most significant food leguminous crops in the world

(CIAT, 2001). It is grown by most small-holder farmers in the eastern African countries for home consumption as

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well as for cash earnings (Hillocks et al., 2006). This region is the most important common bean production area in sub-Saharan Africa and has a high varietal diversity of the crop (Fivawo and Msolla, 2011). Production of common beans in different production areas is hampered by various biotic and abiotic factors which lead to continuous decline of the crop production per unit area (Hillocks et al., 2006). Soil borne diseases caused by either *Fusarium* spp., *Pythium* spp. and or *Rhizoctonia* spp. are biotic constraints to the production of common beans in East African regions; these pathogens act either individually or in a complex manner (Rusuku et al., 1997). Root rot diseases have received little attention until recent years when they became major concerns in East Africa (CIAT, 2003). In Tanzania, limited studies have been conducted regarding this disease and its causative microorganisms. Cultivation of most of the popular commercial common bean genotypes in parts of East Africa is constrained by *Pythium* root rot which results in yield losses of up to 70%. Predictive models have been used to identify new areas where root rots are expected to become a serious problem in Tanzania (Morogoro, Usambara Mountains, parts of Kilimanjaro, Arusha, Mbeya and Kagera), Kenya (Kisii and Nyahururu) and Uganda (Nebi, Apac and parts of Ntungamo) where farmers have already started to encounter root rots, Malawi (the Chitipa Highlands and Shire Highlands), Mozambique (Manica and Lichinga) and Ethiopia (Hararghe) (CIAT, 2003; Wortmann et al., 1998). Therefore, this study aimed at i) ascertaining preliminary information on bean cultivation practices in bean growing parts in Tanzania, ii) in-depth scientific investigation on the incidence and occurrence of *Pythium* root rot disease in relationship with soil pH within some parts of Tanzania.

## MATERIALS AND METHODS

### Collection of diseased plants and soil samples

From surveyed farmers' fields, six infected bean plants showing symptoms of root rot disease were characterized by poor seedling establishment, damping-off, stunting and premature defoliation, deterioration of leaves, plant wilt and death. Symptomatic plants were uprooted using a shovel (Mwang'ombe et al., 2007), put in paper bags and transported to Sokoine University of Agriculture (SUA) Laboratories, Tanzania. Soil samples were also collected at random in the farm at a depth of 0-10 cm with soil auger. Soil pH was determined in a ratio of 1:2.5 soil : water suspension by the potentiometric method (McLean, 1982).

### Isolation of *Pythium* spp. from infected bean plants

Corn meal agar (CMA) growth media (17 g in 1000 ml of distilled water) was autoclaved at 121°C for 15 min; when media was cooled at 40°C, 3 and 0.15 mL of the antibiotics Rifampicin and Pimaricin were added, respectively. Approximately 0.5 to 2 cm of

infected root tissue was cut and rinsed first in 70% ethanol for 30 s then in 2% solution of sodium hypochlorite (NaClO) for 1-2 min and finally rinsed twice in sterilized distilled water. Cut tissues were blotted dry on sterile filter paper, plated on CMA growth media and incubated for 5-7 days at 24°C to allow growth of the pathogen. Based on morphological characteristics of sporangia, oogonia wall, antheridia and oospores are distinctive features of *Pythium* from other root rot pathogens. Purification of *Pythium* culture was carried out by cutting a small piece of the media with mycelia from the edge of a colony and then subcultured onto new growth media. Pure isolates were transferred to potato dextrose agar (PDA) slants and after 14 days were stored at -20°C.

### DNA extraction

Prior to extraction, pure isolates of *Pythium* were reactivated by sub-culturing on PDA growth media and incubated at 24°C for 14 days to allow massive production of mycelia. DNA was extracted from mycelia of *Pythium* using the protocol developed by Mahuku (2004).

### Polymerase chain reaction (PCR)

The internal transcribed sequence (ITS) region was amplified using universal primers ITS1 and ITS4. A reaction volume of 50 µL containing 23.0µL nuclease free water, 25.0 µL of EconoTaqPLUS GREEN 2X Master, 0.5 µL of each primer (10µM) [ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3')] and 1.0 µL of DNA template (Lucigen Corporation 2505 Parmenter St, Middleton, WI 53562 USA) was used. Amplification conditions were achieved in a BIO RAD My Cycler thermal cycler programmed for initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 1 min. At the end of amplification reaction, a final extension step was accomplished at 72°C for 10 min. PCR products attained were run at 1% agarose gels dissolved in 1x TAE (Tris-Acetate EDTA buffer) concentration as the running solution followed with post staining of ethidium bromide (0.5 µg/ml). Electrophoretic migration was carried out for 1 h electrophoresed at 100 V. The amplified products were visualized and photographed under ultraviolet (UV) light. A 100 bp EZ Load molecular ruler (Bio-Rad Laboratories, Inc. CA, USA) was used to estimate the size of PCR products.

### Sequencing, identification and phylogenetic analysis

PCR products with a size of 450 bp and above were sent to Beckman Coulter Company and 43 PCR products were subjected to single pass sequencing (Beckman Coulter Genomics, Inc. Danvers, MA USA). ITS sequences of *Pythium* isolates were compared with ITS sequences of known *Pythium* species available in the GenBank database by performing nucleotide blast search at the National Center for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov/blast.cgi>). The MEGA 6 software was used for phylogenetic analysis (Tamura et al., 2013).

### Statistical analysis

The data collected from the survey was summarized using descriptive statistics such as means, frequencies, percentages and cross tabulations were used to establish the strength of association

**Table 1.** Relative percentages of common bean cultivars grown in Lushoto and Mbozi districts.

	Common bean cultivars	Frequency	Percentage	Cumulative percent
1	Rozikoko	21	25.00	25.00
2	Soya	20	23.80	48.80
3	Njano round	20	23.80	72.60
4	Njano ndefu	9	10.60	83.30
5	Nyeupe ndogo	4	4.80	88.10
6	Kablanketi	3	3.60	91.70
7	JKT	2	2.40	94.10
8	Uyole 2003	2	2.40	96.50
9	Mchanganyiko (Mixed cultivars)	1	1.20	97.70
10	Kibumburi	1	1.20	98.90
11	Selian 94	1	1.20	100.00

**Table 2.** Categories of root rot symptoms in farmers' field.

	Observed symptoms	Frequency	Percentage
1	Wilting, yellowing, water soaked roots and spongy discoloration	34	40.4
2	Dropping yellow leaves, stunted growth and poor germination	30	35.8
3	Death of roots and emerging adventitious roots	16	19.0
4	Water soaked stem extended to hypocotyl	4	4.9

between variables (SPSS Inc., Chertsey, England) (Steel et al., 1997).

## RESULTS

### Root rot distribution

Root rot disease severity was identified from selected hot spot areas, using 84 samples collected which include 43 samples from Lushoto district (five ward locations; Lushoto, Ubiri, Lukozi, Gare and Kwemashai) and 41 samples from Mbozi district (four ward locations; Ruanda, Igamba, Mlowo and Myovizi).

### Production practices of common beans producers within the sampling area

Most of the farmers (63.0%) practiced sole cropping of beans, 31.0% mixed cropping and 6.0% intercropping. Corn (22.7%) was the major crop for either mixed or intercropping systems and other minor crops were banana (4.8%), cassava (2.5%) Irish potatoes (2.5%) and coffee (4.8%). Most farmers (52.4%) kept their own seeds after the season which are used for planting in subsequent cropping season, (32.1%) bought seeds from the local markets, 3.6% got seeds from neighbors, 6.0%

from Agro-dealers and 6.0% from Research Centers. This study showed that no fertilizer was applied by 92.9% of the farmers unless planted in association with corn (7.1%) in the same field. Due to marketability and food sources, the following common bean cultivars were found to be preferred by farmers in Mbozi and Lushoto districts; Soya (23.8%), Njano round (23.8%), Rozikoko (25.0%), Njano ndefu (10.6%), Kablanket (3.6%), Uyole 2003 (2.4%) and JKT (2.4%), Kibumburi (1.2), Selian 94 (1.2) and mixed cultivars (1.2%) (Table 1).

### Occurrence of root rot symptoms in farmers' field

Deterioration of the leaves, wilting, water soaked roots and spongy with discolored cavities dominated in several fields at a frequency of 40.4%, dropping of yellow leaves, stunted growth, uneven growth and poor germination was reported in 35.8% of samples, death of roots and emergence of adventitious roots above the dead root parts and extended brownish color in 19.0% of the samples, water soaked stem extended to hypocotyl of bean seedlings in 4.8% of the samples (Table 2). About 50.0% of respondents indicated that they had observed the above symptoms in their fields in all the seasons, 35.7% observed these symptoms before the 2012 seasons, 8.3% during the 2013 season, and 6.0% throughout the 2014 season (Table 3).

**Table 3.** Period of occurrence of symptoms in farmers' field.

No.	Duration symptoms seen	Frequency	Percent
1	Before 2012 season	30	35.7
2	2013 season	7	8.3
3	2014 season	5	6.0
4	All over seasons	42	50.0

### Morphological and molecular characterization of isolated *Pythium* species and their distribution

Distinctive features of *Pythium*: oogonial wall, oospores, antheridia and sporangia were observed in purified isolates (Figure 1). Forty three PCR product samples with a size of 450 bp and above showed banding patterns and was sequenced for phylogenetic classification according to their respective molecular sequences of the ribosomal fragments (Figure 2). Eleven different *Pythium* species were identified after single pass sequencing; *Pythium aphanidermatum* (31.25%) and *Pythium splendens* (28.13%) being widely distributed in the entire surveyed area. Other species confirmed include: *Pythium ultimum* (6.25%), *Pythium attrantheridium* (6.25%), *Pythium graminicola* (6.25%), *Pythium oligandrum* (6.25%), *Pythium dissotocum* (3.13%), *Pythium irregurale* (3.13%), *Pythium camurandrum* (3.13%), *Pythium paroecandrum* (3.13%) and *Pythium acanthophoron* (3.13%) (Table 4).

### Relationship of Soil pH and disease occurrence

The pH of soil samples collected in the study areas ranged between 5.03 – 6.41 of which 23 isolates were found in pH range of 5.03 – 5.95 and 9 isolates in soil pH range of 6.05 – 6.41. None of the soils had pH higher than 6.5 indicating that, these soils are acidic. Though, *P. splendens* and *P. aphanidermatum* were found in both low and higher ends of the acidic spectrum, *P. splendens* was found in most soils with lower acidic pH values of between 5.03 – 6.12; while *P. aphanidermatum* was found in mostly soils with higher pH values of between 5.41 – 6.41 (Table 4).

### Phylogenetic relationship of *Pythium* spp.

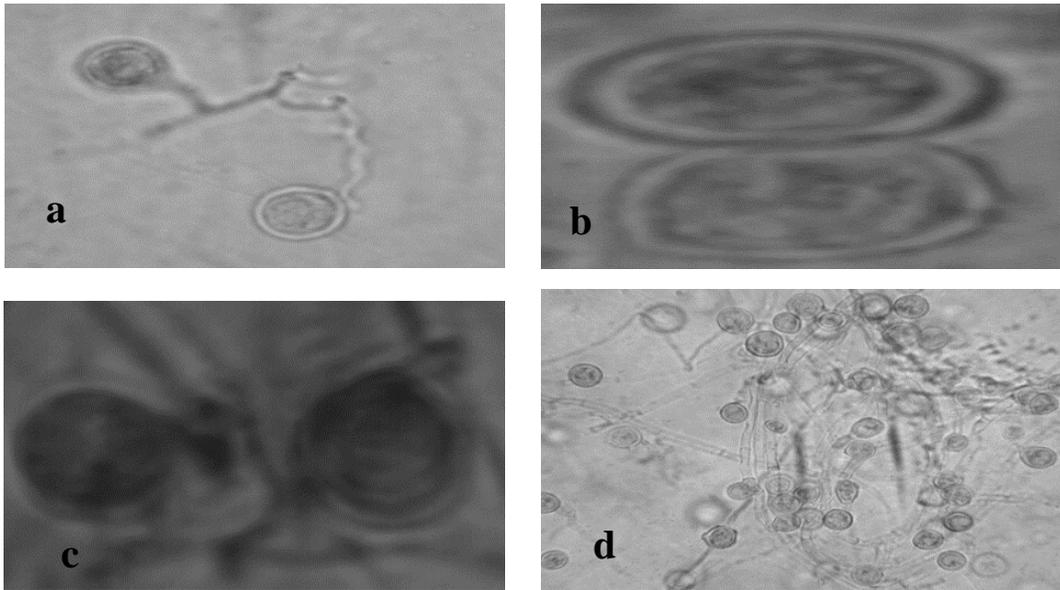
Five clusters with ten sub-clusters showed diversity and homogeneity of species within geographical location (Figure 3). Cluster specific sequences were dispersed over the ITS regions and contributed to the divergence between clusters and convergence between sub-clusters. In cluster I, convergence was observed between *P. aphanidermatum* and other species, in particular *P. dissotocum*, *P. acanthophoron* and *P. oligandrum*

although they originated from different geographical locations. In cluster II, *P. attrantheridium*, *P. paroecandrum*, *P. irregurale* and *P. graminicola* were clustered together due to their close relationships. *P. splendens* and *P. aphanidermatum* in cluster III were closely related despite their origin. Likewise, alignments revealed that, *P. aphanidermatum*, *P. ultimum* and *P. oligandrum* were closely clustered together due to their similar origin in Lushoto district under cluster IV. *P. camurandrum* is the only isolate having distant relationship from other *Pythium* species.

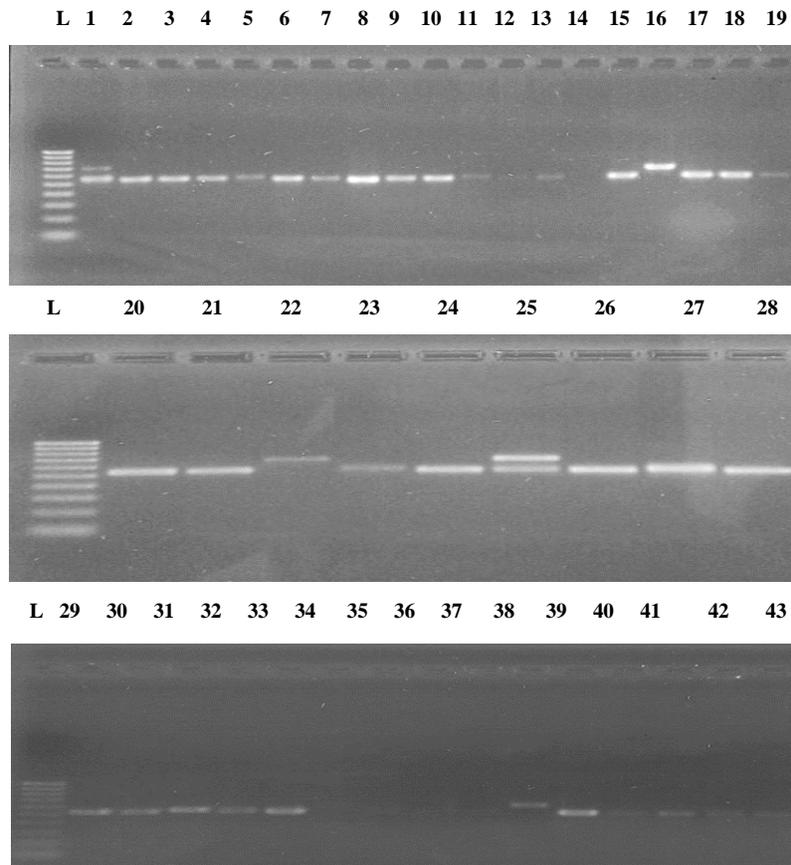
### DISCUSSION

According to the survey conducted in this study, similar root rot symptoms were observed as described by previous findings (Abawi et al., 2006; Agrios, 2005; Buruchara et al., 2010). More farmers use farm saved seeds in which survival structures of the pathogen can be stored together with the seeds and when planted they germinate together and lead to infection and development of the disease. Seed rot and pre-emergence damping-off normally reduce germination rates of planted cultivars due to infection caused by *Pythium* species (Xi et al., 1995). Sole cropping of bean was found to be the dominant system of cultivation as compared to mixed cropping and intercropping due to short rainy season. Previous studies in Uganda showed the incidence of *Pythium* root rot disease in mixed cropping systems. Potatoes, sorghum, maize and peas were found to be susceptible and infected by *Pythium* when intercropped with beans and the cultivation of common beans in mixed cropping systems with other crop species. This partly contributed to beans root rot epidemics and sorghum and peas were found to be the alternative hosts of the pathogenic *Pythium* species (Gichuru, 2008). Studies conducted in Japan showed prevalence of *P. paroecandrum*, *P. spinosum* and *P. ultimum* infecting common beans in rotation plots rather than in sole bean cropping plots because of inoculum build up from other alternate crop prone to root rot (Kageyama, 1981).

Soil pH influences some life cycle stages of *Pythium* species particularly during formation of oospores and sporangia (Martin and Loper, 1999). This study showed that more incidences of *Pythium* pathogen were found between soil pH of 5.1 to 5.6 and less incidence occurred between 6.1 to 6.5 pH; these soils are classified as strongly/moderately acid and slightly acidic, respectively (Soil Survey staff, 1993). Soil pH affects composition of the root exudates, which attract soil borne pathogens (Agrios, 2005). The use of liming materials balances the soil pH to neutrality, and the response of liming materials to soil pH increase is because it removes imbalance of nutrients particularly reduction of aluminum and



**Figure 1.** Morphological features of *Pythium* spp. a: Globose sporangia b: Oospores in an oogonium c: antheridial cell in similar configuration d: Scattered sporangia (large bodies with thin walls) and oospores (smaller, rounder bodies with thick wall) (Magnification 100x).



**Figure 2.** Banding patterns for PCR products electrophoresed at 100 V for 1 h. L = EZ molecular Ruler 100 bp, 1-43 = PCR product samples.

**Table 4.** *Pythium* species by geographical location and soil pH.

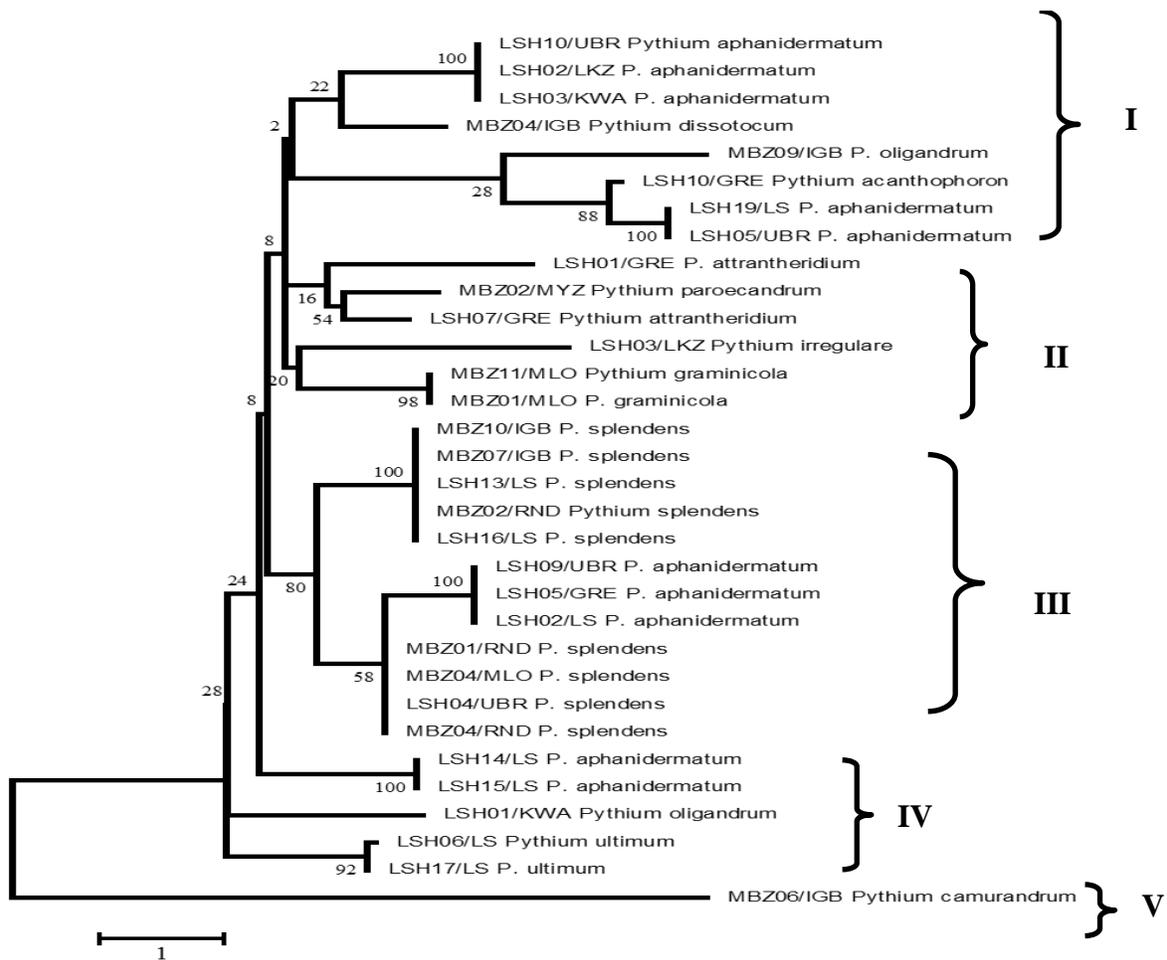
District	Ward	Latitude	Longitude	Altitude (m)	Isolate Codes	<i>Pythium</i> spp.	Soil pH
Lushoto	Ubiri	04°50'03.683"	038°19'48.468"	1220	LSH10/UBR	<i>Pythium aphanidermatum</i>	6.28
Mbozi	Ruanda	09°00'14.639"	033°06'39.954"	1646	MBZ02/RND	<i>Pythium splendens</i>	5.53
Lushoto	Lushoto	04°47'51.312"	038°15'37.853"	1437	LSH06/LS	<i>Pythium ultimum</i>	6.05
Mbozi	Ruanda	08°59'12.642"	033°06'22.878"	1639	MBZ01/RND	<i>P.splendens</i>	6.17
Lushoto	Lushoto	04°47'56.670"	038°17'22.061"	1401	LSH02/LS	<i>P.aphanidermatum</i>	5.74
Lushoto	Lukozi	04°39'52.421"	038°17'32.591"	1805	LSH03/LKZ	<i>Pythium irregurale</i>	5.56
Mbozi	Igamba	08°59'26.298"	032°55'56.771"	1634	MBZ10/IGB	<i>P.splendens</i>	5.03
Lushoto	Kwai	04°42'00.906"	038°20'15.575"	1615	LSH01/KWA	<i>Pythium olingandrum</i>	5.83
Lushoto	Lukozi	04°39'57.054"	038°16'59.574"	1754	LSH02/LKZ	<i>P.aphanidermatum</i>	5.90
Mbozi	Mlowo	09°02'36.906"	032°57'52.422"	1582	MBZ04/MLO	<i>P.splendens</i>	6.14
Lushoto	Lushoto	04°48'08.010"	038°18'37.996"	1526	LSH19/LS	<i>P.aphanidermatum</i>	5.41
Lushoto	Lushoto	04°46'40.601"	038°17'19.445"	1523	LSH14/LS	<i>P.aphanidermatum</i>	5.92
Lushoto	Lushoto	04°47'58.421"	038°18'21.840"	1418	LSH17/LS	<i>P.ultimum</i>	5.65
Mbozi	Igamba	08°59'20.006"	032°55'07.835"	1630	MBZ07/IGB	<i>P.splendens</i>	5.07
Lushoto	Ubiri	04°50'05.568"	038°19'48.155"	1213	LSH09/UBR	<i>P.aphanidermatum</i>	6.08
Lushoto	Lushoto	04°47'56.172"	038°18'34.830"	1457	LSH16/LS	<i>P.splendens</i>	5.78
Mbozi	Mlowo	09°04'33.034"	032°58'59.405"	1633	MBZ11/MLO	<i>Pythium graminicola</i>	6.04
Mbozi	Igamba	08°58'59.915"	032°54'39.630"	1628	MBZO4/IGB	<i>Pythium dissotocum</i>	5.95
Lushoto	Lushoto	04°46'32.190"	038°17'18.702"	1593	LSH13/LS	<i>P.splendens</i>	5.84
Mbozi	Myovizi	09°00'15.810"	033°01'58.020"	1642	MBZ02/MYZ	<i>Pythium paroecandrum</i>	5.42
Lushoto	Gare	04°47'17.298"	038°20'54.732"	1414	LSH07/GRE	<i>Pythium attrantheridium</i>	5.35
Lushoto	Gare	04°47'56.598"	038°20'40.728"	1423	LSH01/GRE	<i>P.attrantheridium</i>	5.31
Lushoto	Gare	04°47'51.551"	038°20'09.810"	1538	LSH10/GRE	<i>Pythium acanthophoron</i>	6.26
Mbozi	Mlowo	09°02'21.245"	032°58'32.876"	1623	MBZ01/MLO	<i>P.graminicola</i>	5.65
Lushoto	Kwai	04°40'00.906"	038°20'12.575"	1623	LSH03/KWA	<i>P.aphanidermatum</i>	5.78
Lushoto	Lushoto	04°46'40.308"	038°17'18.240"	1541	LSH15/LS	<i>P.aphanidermatum</i>	5.67
Mbozi	Igamba	08°59'50.628"	032°55'44.483"	1635	MBZ09/IGB	<i>P.ollingandrum</i>	5.74
Lushoto	Ubiri	04°50'08.178"	038°19'19.446"	1238	LSH04/UBR	<i>P.splendens</i>	5.81
Mbozi	Ruanda	09°01'45.329"	033°06'15.282"	1712	MBZ04/RND	<i>P.splendens</i>	6.12
Mbozi	Igamba	08°58'19.362"	032°54'24.263"	1626	MBZ06/IGB	<i>Pythium camurandrum</i>	5.89
Lushoto	Gare	04°47'25.644"	038°20'40.476"	1481	LSH05/GRE	<i>P.aphanidermatum</i>	5.47
Lushoto	Ubiri	04°50'00.738"	038°19'16.272"	1226	LSH05/UBR	<i>P.aphanidermatum</i>	6.41

manganese toxicities which provides calcium ions to counteract its deficiency (Biswas and Mukherjee, 1994; Nekesa et al., 2005). Also, soil pH affects the availability of nutrients to the plant which are needed for strong cell walls and resistance to fungal infestations. For instance, high levels of available calcium in more alkaline soils have been implicated in the resistance to root diseases caused by *Pythium* species (Paulitz, 2002).

Ribosomal DNA sequences of the ITS region identified eleven *Pythium* species; *P. aphanidermatum* and *P. splendens* which were the most widely distributed species in the study locations. Other species confirmed include *P. dissotocum*, *P. ultimum*, *P. irregurale*, *P. camurandrum*, *P. attrantheridium*, *P. graminicola*, *P. paroecandrum*, *P. acanthophoron* and *P. oligandrum*. Previous studies conducted by Mukalazi (2004) and

Nzungize et al. (2011) in Uganda and Rwanda, respectively identified some similar species that cause root rot disease. However, none of these identified eleven species have been previously studied and documented in Tanzania. Therefore, this study is the first to identify these *Pythium* spp. in common bean cultivation in Tanzania.

There was no association between the geographic distribution and identification of *Pythium* species within the collection area. For instance, *P. aphanidermatum* was found in altitudes of 1213, 1526 and 1754 m above sea level which is similar to the study conducted by Nzungize et al. (2011) that *P. vexans* was found in the highest number of districts where common beans were grown and this species was identified in low, intermediates and high altitudes of 900-1400, 1400-1650 and 1650-2300 m,



**Figure 3.1** Evolutionary phylogenetic relationship of *Pythium* spp. based on ITS ribosomal DNA sequences aligned by ClustalW and constructed by maximum likelihood tree. First three letters representing district, a numeral represent sampling number within a ward and last letters represent wards (LSH=Lushoto, MBZ=Mbozi, LKZ=Lukozi, KWA=Kwai, LS=Lushoto, IGB=Igamba, UBR= Ubiri, MLO= Mlowo, MYZ= Myovizi, GRE=Gare, RND=Ruanda).

respectively. In previous findings, similar studies identified *P. aphanidermatum* as a causal agent of root rot and crown necrosis of mature bean plants in Oman. This species was identified as the most aggressive and pathogenic species in the genus; it also has a wide host range that causes many economically important root rot disease (Al-Mahmooli et al., 2015; Ben Yephet and Nelson, 1999; Haritha et al., 2010). The most common species of *Pythium* that cause plant diseases of economic importance in Florida are *Pythium myrtilum* and *P. aphanidermatum* and other species of *Pythium* that are sometimes associated with dysfunctional plants were *P. splendens* and *P. irregulare*. Also, *P. splendens* was identified from *Eucalyptus grandis* in northern Natal in South Africa and from soybean and corn in Ohio, USA (Dorrance et al., 2004; Kucharek, 2000; Linde et al., 1994). In eastern Washington, several species including

*P. atrrantheridium*, *P. irregulare* and *P. paroecandrum* were identified by using a real time PCR using collected soil samples (Li et al., 2014; Schroeder et al., 2006). This study also identified *P. oligandrum* from farmers' field within mono cropping system of common beans (Gichuru, 2008).

The phylogenetic analysis indicated diversity and similarities obtained from the alignment analysis of the ITS sequenced data using species-specific primers of 5.8S rDNA sequences. Specific proportional studies of the nucleotide sequences of rDNA genes provide a significant way of analyzing phylogenetic relationships over a wide range of taxonomic specie levels in fungal and non-fungal groups (Berbee et al., 1995; Harlton et al., 1995).

The spread of *Pythium* spp. occurs mostly through the movement of infested soil and plant materials by irrigation

water, wind, farm equipment or animals. Heavy use of nitrogenous fertilizers and removal from farm products like plant residues after harvesting take alkaline nutrients off, tend to accelerate the rate of soil acidification and make favorable conditions for *Pythium* development. Therefore, farmers are advised to leave plant residues in the field after harvesting and use agricultural liming materials so as to increase soil pH from acidity to neutrality. This study provided information on the occurrence and distribution of *Pythium* root rot disease in two districts of Tanzania where common beans are grown.

### Conflict of interests

The authors have not declared any conflict of interest.

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*Full Length Research Paper*

# Effect of poultry manure treated and untreated with effective microorganisms on growth performance and insect pest infestation on *Amaranthus hybridus*

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Poor soil fertility is a major cause of low yield of amaranth in Nigeria. Optimum productivity of the vegetable is also constrained by insect pests that cause reduction in yield and quality. Incorporation of effective microorganisms (EM) into organic matter is capable of positively influencing decomposition and mineralization. The present study assessed the effects of poultry manure treated and untreated with effective microorganisms on growth performance, yield and insect pest infestation on *Amaranthus hybridus*. The experimental design used was a randomized complete block design (RCBD) with 3 replications. The treatments consist of poultry manure treated with effective microorganism activated solution (PM + EMAS), poultry manure only (PM), and the control (C). Data were collected on shoot height, stem girth, number of leaves per plant, leaf area, total fresh leaf weight and mean pest number. The results from this study shows that incorporation of effective microorganisms into poultry manure significantly increased shoot height, stem diameter, leaf number, leaf area and fresh leaf weight. A significantly ( $P < 0.05$ ) higher fresh weight (36.85 kg) of *A. hybridus* was obtained in plots treated with PM+EMAS. This was followed by plots treated with PM only (25.08 kg). The control had a significantly least fresh weight of 14.21 kg. Six insect species from 4 orders and 5 families were encountered on *A. hybridus* during the study period. They include *Zonocerus variegatus*, *Podagrica* spp., *Hymenia recurvalis*, *Nezara viridula*, *Psara bipunctalis* and *Sylepta derogata*. *Hymenia recurvalis* was the most prevalent pest recorded in the control plot (16.4). Generally, fewer number of pest species were observed in plots treated with PM+EMAS. The use of effective microorganisms in organic farming is a viable tool for curbing the menace arising from the use of synthetic fertilizers and pesticides.

**Key words:** *Amaranthus hybridus*, poultry manure, effective microorganisms, growth parameters, yield, pest infestation.

## INTRODUCTION

Red amaranth (*Amaranthus hybridus*) is one of the cheapest and widely cultivated leafy vegetables in

Nigeria. Amaranth leaves are rich in calcium, phosphorus, folic acid, potassium, iron and vitamins A, B and C (AVRDC, 2003; Okpara et al., 2013; Oyedeji et al., 2014).

Nigeria ranks as the largest producer and consumer of *Amaranthus* spp. in Africa (Raemaekers, 2001). The world average yield of amaranth is estimated at 14.27 t ha<sup>-1</sup> (FAO, 2007). The yield per hectare in Nigeria is low (7.60 t ha<sup>-1</sup>) compared to what obtains in the United States (77.27 t ha<sup>-1</sup>). Low soil fertility is a major cause of low yield of amaranth in Nigeria (Fasina et al., 2015; Shehu et al., 2015). Optimum productivity of the vegetable is also constrained by several insect pests that cause reduction in yield and quality (Aderolu et al., 2013). Insect pest infestation alone has been reported to account for 20 to 60% pre-harvest losses in vegetables in the developing countries (Sithantham et al., 2003).

Most tropical soils exhibit rapid depletion of organic matter which causes an increase in the use of synthetic fertilizers that poses serious threat to soil and human health (Okito et al., 2004). To obtain high yield of amaranth, there is a great need to augment the nutrient status of the soil to meet the crop need (Dauda et al., 2005a). One of the ways of increasing the fertility of the soil is by boosting its nutrient content with organic matter such as poultry manure.

Poultry manure contains more plant nutrients than all other organic manures (Ali, 2005). Owing to its high organic matter content combined with available nutrients needed for improving plant growth, it is widely utilized as an excellent soil amendment. The manure is cheap and readily available. Decomposed poultry manure stimulates microbial activities which contribute to soil fertility restoration (Rahman, 2004).

Incorporation of effective microorganisms (EM) into organic matter is capable of positively influencing decomposition and mineralization. Effective microorganisms consist of mixed cultures of beneficial and naturally occurring microorganisms that can be applied as inoculants to increase the microbial diversity of soil and plant (Muthaura et al., 2010). Research has shown that the inoculation of EM cultures to the soil plant ecosystem can improve soil quality, soil health and the growth, yield and quality of the crops (Kengo and Hui-lian, 2000). This ultimately leads to increase in the microbial diversity and activity in soils and plants (Zimmermann and Kamukuenjandje, 2008). Inoculation of EM cultures to the soil and plant ecosystem can improve soil quality and the growth, yield, and quality of crops. More importantly, EM helps to enhance crop quality and health to resist pest attack by improving

their strength to withstand infestation. The present study therefore assessed the effect of poultry manure treated and untreated with effective microorganisms on growth performance, yield and insect pest infestation on *A. hybridus*.

## MATERIALS AND METHODS

### Study site

The research work was carried out at the Teaching and Research Farm of Landmark University, Omu-Aran, Kwara State, Nigeria. The site lies between latitude 8°8' N and longitude 5°6' E of the equator. Annual rainfall ranges between 600 and 1500 mm with a distinct dry season from December to March (Ilorin Meteorological Bulletin, 2003). The mean annual temperature varies between 28 and 34°C.

### Experimental design and treatment application

The land was cleared, ploughed and harrowed following which vegetable beds were prepared. A total land area of 40 m<sup>2</sup> was partitioned into nine plots, with each plot (vegetable bed) measuring 1 m × 3 m. Each plot was demarcated into three rows and separated from the adjacent bed by 0.5 m alley.

Fresh poultry manure (PM) collected from the Poultry Section of Landmark University Teaching and Research Farm was cured by allowing it to decompose for four weeks. It was thoroughly incorporated into three randomly selected vegetable beds at the rate of 30 kg ha<sup>-1</sup> two weeks before planting and watered regularly to enhance mineralization.

Effective microorganism activated solution (EMAS) procured from Songhai Centre, Porto-Novo, Benin Republic was mixed with the decomposed poultry manure at the recommended rate of 5 ml per 2 kg of PM. This was applied at 30 kg ha<sup>-1</sup> into three randomly selected beds two weeks before planting. The beds were watered regularly to enhance the mineralization of the poultry manure and the EMAS.

Seeds of *A. hybridus* obtained from the Teaching and Research Farm of Landmark University, Omu-Aran were drilled into the top-soil at a depth of 1 cm in each treatment. The experiment was laid out in a randomized complete block design (RCBD) with 3 replications. The treatments consist of PM treated with effective microorganism activated solution (PM + EMAS) and PM only. A control treatment without addition of PM or EMAS was also set up. Irrigation was carried out twice daily; morning and evening.

Data collection on growth parameters commenced two weeks after planting and was carried out at every 5 days. Ten plants were selected randomly from each row for data on shoot height, stem girth, number of leaves per plant and leaf area. Yield assessment was based on total fresh leaf weight at six weeks after planting. Shoot height was measured using a meter rule while stem diameter was determined with a vernier caliper. Leaf area expansion was determined according to Jose et al. (2000), using the formula:

$$A_L = 0.73 (L_L \times W_L)$$

Where,  $A_L$  = leaf area,  $L_L$  = leaf length and  $W_L$  = maximum width

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**Table 1.** Effect of different treatments (poultry manure, PM; poultry manure with effective microorganism activated solution, PM+EMAS; and control, C) on the shoot height (cm) of *Amaranthus hybridus* at different number of days after planting (DAP).

Treatment	15 DAP	20 DAP	25 DAP	30 DAP	35 DAP
PM	8.26 <sup>b</sup>	10.96 <sup>b</sup>	17.45 <sup>b</sup>	21.17 <sup>b</sup>	23.68 <sup>b</sup>
PM +EMAS	10.53 <sup>a</sup>	13.83 <sup>a</sup>	25.57 <sup>a</sup>	35.02 <sup>a</sup>	39.27 <sup>a</sup>
C	7.27 <sup>c</sup>	8.13 <sup>c</sup>	11.28 <sup>c</sup>	15.63 <sup>c</sup>	17.23 <sup>c</sup>

Values followed by the same letter within the same column are not significantly different ( $p \leq 0.05$ ).

**Table 2.** Effect of different treatments (poultry manure, PM; poultry manure with effective microorganism activated solution, PM+EMAS; and control, C) on the number of leaves of *A. hybridus* at different number of days after planting (DAP).

Treatment	15 DAP	20 DAP	25 DAP	30 DAP	35 DAP
PM	5.79 <sup>a</sup>	7.48 <sup>b</sup>	10.10 <sup>b</sup>	13.70 <sup>b</sup>	16.49 <sup>b</sup>
PM +EMAS	6.35 <sup>a</sup>	9.80 <sup>a</sup>	13.64 <sup>a</sup>	17.30 <sup>a</sup>	22.37 <sup>a</sup>
C	3.69 <sup>c</sup>	4.63 <sup>c</sup>	6.49 <sup>c</sup>	8.64 <sup>c</sup>	10.80 <sup>c</sup>

Values followed by the same letter within the same column are not significantly different ( $p \leq 0.05$ ).

measured for all leaves on each plant

Insect collection was carried out very early in the morning and late in the evening. The upper and lower leaf surfaces of the vegetable in each treatment were carefully examined for insects and were collected by hand (insects on the plant surface) or with a sweep net (flying insects). They were put into vials, labeled according to the treatment in which they were found and taken to Landmark University Laboratory, where they were classified, counted and recorded according to treatment.

### Statistical analysis

Data collected were subjected to Analysis of Variance (ANOVA) and means separated using Duncan's multiple range test (DMRT) at 5% probability level.

## RESULTS

### Shoot height

The effects of PM and PM+EMAS treatments on shoot height of *A. hybridus* are shown in Table 1. At fifteen days after planting (DAP), plants in plots treated with PM + EMAS had a significantly ( $P \leq 0.05$ ) higher mean shoot height (10.53 cm), followed by PM plants (8.26 cm). Height of C-plants was significantly lower (7.27 cm). Similar trends of result were also recorded at 20, 25, 30 and 35 DAP.

### Number of leaves per plant

At 15 DAP, there was no significant difference in the

number of leaves produced by the vegetable in PM and PM+EMAS plots, although numerically higher values were recorded in the latter (Table 2). The control had significantly ( $P \leq 0.05$ ) lower number of leaves (3.69) among the treatments at 15 DAP. At 20, 25, 30 and 35 DAP, significantly higher numbers of leaves were recorded in PM + EMAS plots, followed by PM only. Significantly lowest leaf numbers were observed in C-plots.

### Stem diameter

The effect of PM and PM+EMAS treatments on stem girth of *A. hybridus* is shown in Table 3. At 15 DAP, a significantly ( $P \leq 0.05$ ) higher stem girth (0.94 cm) was recorded in plots treated with PM + EMAS. This was followed by a stem diameter of 0.67 cm observed in PM plots while significantly least stem girth (0.43 cm) was observed in the control plot. Similar trend of result was also observed at 20, 25, 30 and 35 DAP.

### Leaf area

At 15 days DAP, plants in plots treated with PM + EMAS had a significantly ( $P \leq 0.05$ ) higher leaf area (21.56 cm<sup>2</sup>), followed by plants in plots treated with PM only (18.31 cm<sup>2</sup>). The control had a significantly least leaf area (13.47 cm<sup>2</sup>). Similar trends of result were also recorded at 20, 25, 30, and 35 DAP.

**Table 3.** Effect of different treatments (poultry manure, PM; poultry manure with effective microorganism activated solution, PM+EMAS; and control, C) on the stem girth (cm) of *A. hybridus* at different number of days after planting (DAP).

Treatment	15 DAP	20 DAP	25 DAP	30 DAP	35 DAP
PM	0.67 <sup>b</sup>	0.96 <sup>b</sup>	1.37 <sup>b</sup>	1.75 <sup>b</sup>	2.03 <sup>b</sup>
PM +EMAS	0.94 <sup>a</sup>	1.17 <sup>a</sup>	2.03 <sup>a</sup>	2.61 <sup>a</sup>	2.92 <sup>a</sup>
C	0.43 <sup>c</sup>	0.57 <sup>c</sup>	0.82 <sup>b</sup>	0.89 <sup>c</sup>	1.75 <sup>c</sup>

Values followed by the same letter within the same column are not significantly different ( $p \leq 0.05$ ).

**Table 4.** Effect of different treatments (poultry manure, PM; poultry manure with effective microorganism activated solution, PM+EMAS; and control, C) on the leaf area (cm<sup>2</sup>) of *A. hybridus* at different number of days after planting (DAP).

Treatment	15 DAP	20 DAP	25 DAP	30 DAP	35 DAP
PM	18.31 <sup>b</sup>	20.76 <sup>b</sup>	24.53 <sup>b</sup>	36.86 <sup>b</sup>	44.32 <sup>b</sup>
PM +EMAS	21.56 <sup>a</sup>	27.40 <sup>a</sup>	32.92 <sup>a</sup>	48.36 <sup>a</sup>	57.06 <sup>a</sup>
C	13.47 <sup>c</sup>	15.18 <sup>c</sup>	16.77 <sup>c</sup>	20.34 <sup>c</sup>	25.72 <sup>c</sup>

Values followed by the same letter within the same column are not significantly different ( $p \leq 0.05$ ).

**Table 5.** Effect of different treatments (poultry manure, PM; poultry manure with effective microorganism activated solution, PM+EMAS; and control, C) on the total fresh weight (kg) of *A. hybridus* at different number of days after planting (DAP).

Treatment	Mean fresh weight (kg)
PM	25.08
PM+EMAS	36.24
C	14.21

Values followed by the same letter within the same column are not significantly different ( $p \leq 0.05$ ).

### Fresh leaf weight

The effect of PM and PM+EMAS treatments on the total leaf fresh weight of *A. hybridus* is shown in Table 4. A significantly ( $P \leq 0.05$ ) higher fresh weight (36.85 kg) of *A. hybridus* was obtained in plots treated with PM + EMAS. This was followed by plots treated with PM only (25.08 kg). The control had a significantly least fresh weight of 14.21 kg (Table 5).

### Insect pest infestation

A total of 6 insect species from 4 orders and 5 families were encountered on *A. hybridus* during the study period (Table 6). They include *Z. variegatus*, *Podagrica* spp., *H. recurvalis*, *N. viridula*, *Psarabipunctalis* and *S. derogata*. *H. recurvalis* was the most prevalent pest recorded in the control plot (16.4). Generally, fewer number of pest

species were observed in plots treated with PM + EMAS.

### DISCUSSION

The results from this study show that incorporation of effective microorganisms into poultry manure significantly increased shoot height, stem diameter, leaf number, leaf area and fresh leaf weight. Increase in number of leaves in plants provided with adequate nutrition is a common occurrence in plants and can be attributed to increase in the photosynthetic activity of the plants (Muthaura et al., 2010).

The higher number of leaves, shoot height, leaf area, increased yield as well as reduced pest infestation on *A. hybridus* treated with EM recorded in this trial is in consonance with the findings of Reddy and Giller (2008) that reported successful control of sucking insects on legumes and cucurbits with EM preparation as well as improved growth in the leaves and stems of crops sprayed with different EM preparations, leading to yield increases of 15%.

Soil health is the key to producing good yield of crops. Vegetable crops have high nutrient requirement and application of nitrogen, a major component of poultry manure improves the yield of amaranth (Rahman, 2004). Research has shown that applying EM to the soil and plant ecosystem improves soil quality, soil health, and the growth, yield, and quality of crops. Application of EM to organic matter helps to improve biological activity through effective organic matter recycling. The organic matter recycled builds humus, the food for soil and plants. This

**Table 6.** Effect of different treatments (poultry manure, PM; poultry manure with effective microorganism activated solution, PM+EMAS; and control, C) on the mean pest number on *A. hybridus*.

Treatment	Common name	Order	Family	Scientific name	Mean number
PM					4.2
EMAS + PM	Grasshopper	Orthoptera	Acrididae	<i>Zonocerus variegatus</i>	1.8
Control					8.3
PM					7.8
EMAS + PM	Flea beetles	Coleoptera	Chrysomelidae	<i>Podagricasp.</i>	1.3
Control					6.5
PM					4.8
EMAS + PM	<i>Amaranthus</i> caterpillar	Lepidoptera	Crambidae	<i>Hymenia recurvalis</i>	0.7
Control					16.4
PM					1.8
EMAS + PM	Green vegetable bug	Heteroptera	Pentatomidae	<i>Nezaraviridula</i>	0.7
Control					4.6
PM					1.5
EMAS + PM	Leaf webber	Lepidoptera	Pyralidae	<i>Psarabipunctalis</i>	0.3
Control					4.1
PM					0.7
EMAS + PM	Cotton leaf roller	Lepidoptera	Pyralidae	<i>Syleptaderogata</i>	-
Control					3.3

process ultimately enhances nitrogen fixation, improves mycorrhizal activity, and hence, leads to more effective nutrient availability.

When effective combination of microorganisms makes contact with organic materials such as poultry manure, beneficial substances like vitamins, organic acids, minerals and anti-oxidants are secreted (Muthaura et al., 2010). As such, there is transformation in the micro-flora and macro-flora, leading to improvement in restoration of the natural health of the soil. This helps to improve plant growth.

In the present study, seedling emergence commenced three days after sowing in soils treated with EMAS + PM, while germination was observed after five days in the control plots. EM contains many micro-organisms, mainly lactic acid bacteria, yeast and phototrophic bacteria that speed up the decomposition of organic matter to promote germination (Reddy and Giller, 2008). Therefore, the early germination observed could be attributed to the presence of EM.

Vegetable crops are highly susceptible to pests and diseases during the growing phase. Incorporation of EM to organic matter performs two major functions. Firstly, it creates better growing conditions that leads to a stronger and healthier plant. Secondly, it inoculates leaf surfaces with beneficial microbes, thereby excluding pests and pathogens that compete with crops for space. EM acts as an insect repellent by creating a barrier around the plant,

thereby protecting it from insect pest attack (Higa, 1998). Pests are suppressed or controlled through natural processes that enhance the competitive and antagonistic activities of the microorganisms in the EMAS.

## Conclusion

The declining soil fertility, increased soil erosion and increasing food shortage are major factors affecting human health in Africa. Fertilizers are costly and beyond the reach of most resource poor farmers. The use of synthetic agrochemicals and fertilizers has caused adverse effects on human health and the environment. This observation has promoted the need to introduce novel farming methods capable of reducing health risks. The use of effective microorganisms in organic farming is a viable tool for curbing the menace arising from the use of synthetic fertilizers and pesticides.

## Conflict of Interests

The authors have not declared any conflict of interest.

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## Full Length Research Paper

# Mistletoe presence on five tree species of Samaru area, Nigeria

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The infestation of trees by mistletoe within Samaru is very high and alarming and there is little or no records on the type of mistletoe species found parasitic on tree species within this area. In order to document and know the species richness of mistletoe within this region, the study was aimed at determining the different species of mistletoe parasitic on *Albizzia lebeck*, *Citrus grandis*, *Khaya senegalensis*, *Terminalia mantaly* and *Terminalia catappa* within Samaru, Nigeria. The study site was divided into four sampling areas based on the presence of the studied species, and the infection of the trees by mistletoes. In each of the sampling areas, the leaves of mistletoes found parasitic on each of these tree species were collected. The study indicated that from all the sampling areas, *A. lebeck* was infected by six different species of mistletoe: *Tapinanthus dodoneifolius*, *Tapinanthus globiferus*, *Globimetula braunii*, *Globimetula oreophila*, *Englerina lecardii* and *Tapinanthus belvisii*; *C. grandis*, *T. catappa* and *T. mantaly* each had four different species found parasitic on them, and *K. senegalensis* had three different mistletoes species parasitic on it. *A. lebeck* had the highest number of different mistletoe species found parasitic on it while *K. senegalensis* had the lowest. *G. braunii* and *T. globiferus* were the most common mistletoe species found parasitic on all the targeted host trees while *T. dodoneifolius* was found parasitic only on *A. lebeck* and *T. catappa* and *E. lecardii* was found parasitic only on *A. lebeck* and *C. grandis* respectively in the study area. In conclusion, among the studied tree species, *A. lebeck* was the most vulnerable to mistletoe attack in the study area and *G. braunii* and *T. globiferus* were less host specific.

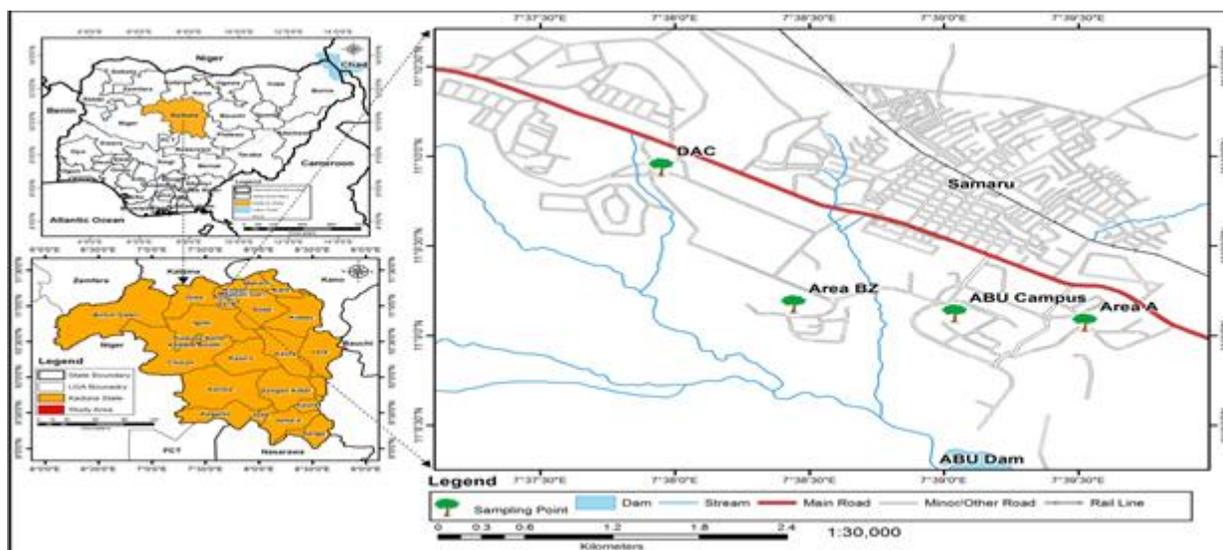
**Key words:** Distribution, host range, mistletoe, Samaru.

## INTRODUCTION

Mistletoe, which consists of about 1400 species around the world, belongs to the kingdom Plantae, subkingdom Tracheobionta, super-division Spermatophyte, division

Magnoliophyta, class Magnoliopsida, subclass Rosidae, order Santales (Judd et al., 2002). Recent phylogenetic studies confirm that mistletoes belong to five distinct

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**Figure 1.** Map of Samaru with the location of sampling areas.

families: Misodendronaceae, Eremolepidaceae, Santalaceae, Viscaceae and Loranthaceae (Der and Nickrent, 2008, Malecot and Nickrent, 2008, Vidal-Russell and Nickrent, 2008). The largest family of this mistletoe is Loranthaceae which has 75 genera and over 900 species (Judd et al., 2002). Among them, six major genera are found in Nigeria, namely: *Tapinanthus*, *Agelanthus*, *Loranthus*, *Globimetula*, *Phragmanthera* and *Englerina*. *Tapinanthus* is far more widespread in the Nigeria savanna (Johri and Bhatnagar, 1972; Omolaja and Gamaye, 1998). Mistletoe, in Yoruba speaking area in Nigeria, it is called 'afomo', in Igbo 'apari' while in Hausa it is called 'kauci' and 'children's matches' in Eastern Cameroon presumably due to the match-like shape of the flower (Oluwole et al., 2013).

All mistletoes are hemi-parasites, bearing evergreen leaves that photosynthesize but depend on their host mainly for water and mineral nutrients (Milius, 2000). These mistletoes grow on a wide range of host trees, and it may reduce their growth and eventually they can kill the trees with heavy infestation.

Seeds of most mistletoe are spread by birds that eat the fruits (Cowles, 1964) or by the wind. The mistletoe seed germinates on the branch of a host tree or shrub and in its early stages of development it is independent of its host. Later it forms a haustorium that penetrates the host tissue and takes water and nutrients from the host plant (Milius, 2000).

Many of these parasitic plants (mistletoes) can simultaneously parasitize many host species. Since different host species may supply a parasite with different resources, a mixture of host species may be superior to a single host alone. Boussium et al. (2004) reported that mistletoe (*T. globiferus*) parasitized 126

species, and believed that it is less specific compared to other mistletoe species. Despite the large host range of the majority of parasitic plants, many also show high levels of host preference. In mistletoe plants, host choice can be considerably influenced with relatively abundant hosts (Norton and Carpenter, 1998; Norton and De Lange, 1999), host characteristics such as branch size, age and height and the duration of association between the host and the parasite (Didier et al., 2009).

It has been observed that in Samaru, the infestation of trees by mistletoe is very high and generates great concern in the local people as these mistletoes result in the reduction of vegetation and fruit production of trees in the region. Also, there is little or no records on the type of mistletoe species found parasitic on tree species within this area. As such, in order to document the number of mistletoe species and record the rate of infestation of trees in this region, this study is aimed at determining the presence of different mistletoe species on some selected trees with medicinal importance within Samaru (Zaria, Nigeria).

## MATERIALS AND METHODS

### Study area

The study area is Samaru, Zaria, Kaduna State (Nigeria) which falls within the Guinea Savannah zone (07°37'22" to 7°40'36" EL, 11°09'14" to 11°10'09" NL). It has a size of 23.46 km<sup>2</sup>. The area was divided into four sampling areas which includes: Areas A, BZ, DAC and ABU main campus with estimated sizes of 0.90, 0.74, 1.15 and 0.56 km<sup>2</sup> respectively based on the presence of *Albizia lebbek*, *Citrus grandis*, *Khaya senegalensis*, *Terminalia mantaly* and *Terminalia catappa* and with the presence of infestation of trees by mistletoes (Figure 1).

### Collection of mistletoes

In each of the sampling areas, three separate plants of each of *A. lebbbeck*, *C. grandis*, *K. senegalensis*, *T. mantaly* and *T. catappa* were randomly chosen based on the heavy infestation of their branches with different species of mistletoe and fresh leaves of the mistletoes found parasitic on it were collected. The samples were taken to the herbarium unit of the Department of Biological Sciences, Ahmadu Bello University (Zaria) for further identification.

### Counting of host plants

Each of the sampling areas was according to its size divided into clusters. Sampling areas A, BZ, DAC and ABU main campus were divided into 34, 27, 20 and 40 clusters respectively and each cluster was 200 m in size. And the number of *A. lebbbeck*, *C. grandis*, *K. senegalensis*, *T. mantaly* and *T. catappa* within each of the clusters was noted, taking note of those infected and uninfected by mistletoes.

## RESULTS

### Infestation of trees by mistletoe

The results revealed that, all the selected tree species were infected in virtually all the sampling areas and there was significant relationship between the infected and uninfected tree species in all areas except *T. catappa* which had insignificant relationship between its infected and uninfected trees in all the sampling areas at  $P < 0.05$  (Table 1). *A. lebbbeck* in sampling area A was the highest in number of trees compared to other areas. However, the *A. lebbbeck* in area BZ had the highest percent (77.27%) of infection whereas the ones in ABU main campus had the least (45.80%) (Table 1).

*K. senegalensis* and *C. grandis* in area A, were the highest in number of trees, however, it had the least percent (29.48 and 56.41% respectively) of infection by mistletoes while those in sampling area BZ had the highest percent (57.71 and 80.00% respectively) compared to those in other areas (Table 1). ABU main campus had the highest number of *T. mantaly* as well as the percentage of those infected by mistletoes (61.33%) whereas in area BZ none of the *T. mantaly* there was infected (Table 1).

Also, ABU main campus had the highest number of *T. catappa*, however, area A had the least percent (25.00%) of those infected by mistletoes whereas those in area BZ had the highest percent (66.67%) of infection (Table 1). All the tree species except *T. catappa*, had significant relationship between the infected and uninfected in each of the sampling areas (Table 1).

### Level of infestation of tree species by mistletoe

In comparison of each tree species from all sampling

areas, *A. lebbbeck* had the highest level of infestation by mistletoes (58.26%) than other tree species. However, the level of the infestation was significantly similar to those of other tree species (Table 1).

### Species of mistletoe identified on the targeted host trees in area A

On *A. lebbbeck*, four different species of mistletoe were identified, include: *G. oreophila*, *G. braunii*, *T. globiferus* and *T. dodoneifolius*. And on *T. catappa* were found two species of mistletoe: *T. dodoneifolius* and *G. braunii*. However, on *C. grandis*, *K. senegalensis* and *T. mantaly* was only found one species of mistletoe in each tree species (*G. braunii*, *T. globiferus* and *G. oreophila*, respectively).

### Species of mistletoe identified on the targeted host trees in area BZ

In area BZ (Figure 1), two species of mistletoe were identified on *A. lebbbeck*, known as *T. belvisii* and *T. dodoneifolius*, and on *C. grandis* and *K. senegalensis*, only *G. oreophila* was identified, where on *T. catappa*, only *G. braunii* was identified. *T. mantaly* was part of the targeted host tree before which was not found in the area (Table 2).

### Mistletoe species identified on the targeted host trees in DAC

From the sampling area DAC (Figure 1), three species of mistletoe identified on *A. lebbbeck* were *E. lecardii*, *T. dodoneifolius* and *G. oreophila*. However, *C. grandis* and *T. mantaly* had two species of mistletoe each (*T. globiferus*, *E. lecardii*, *T. belvisii* and *T. globiferus*, respectively). *T. catappa* and *K. senegalensis* had one species of mistletoe each, which was identified as: *T. belvisii* and *G. oreophila*, respectively (Table 2).

### Mistletoe species identified on the targeted host trees in ABU main campus

In the sampling area ABU (Figure 1), three species of mistletoe (*G. oreophila*, *T. dodoneifolius* and *T. belvisii*) were found parasitic on *A. lebbbeck*. On *T. catappa* and *K. senegalensis*, two species of mistletoe were found on each tree species (*T. globiferus*, *G. braunii* and *G. braunii* and *T. globiferus* respectively). *Citrus grandis* which was one of the targeted host tree was not found in the area (Table 2).

The summary of the observations from all the

**Table 1.** Selected host tree species in each of the sampling areas indicating number of those infected and those uninfected by mistletoes.

Sampling area	Host tree	No. of tree	Infected (%)	Uninfected (%)	X <sup>2</sup>	Df	P- value	Level of infestation of each tree sp from all areas (%)
Area A	<i>A. lebbbeck</i>	837	456 (54.55)	381 (45.57)	48.24	3	0.00	58.26 <sup>a</sup>
ABU main campus		262	120 (45.80)	142 (54.19)				
Area BZ		198	153 (77.27)	45 (22.73)				
DAC		92	51 (55.43)	41 (44.57)				
Total		1389	780 (233.05)	609 (167.06)				
Area A	<i>K. senegalensis</i>	631	186 (29.48)	445 (70.52)	105.36	3	0.00	48.20 <sup>a</sup>
ABU main campus		457	242 (52.95)	215 (47.05)				
Area BZ		454	262 (57.71)	192 (42.29)				
DAC		134	70 (52.24)	64 (47.76)				
Total		1676	760 (192.38)	916 (207.62)				
Area A	<i>C. grandis</i>	39	22 (56.41)	17 (43.59)	8.09	3	0.04	49.10 <sup>a</sup>
ABU main campus		3	0 (0.00)	3 (100.00)				
Area BZ		20	16 (80.00)	4 (20.00)				
DAC		10	6 (60.00)	4 (40.00)				
Total		72	44 (196.41)	28 (203.59)				
Area A	<i>T. mantaly</i>	13	4 (30.77)	9 (69.23)	34.13	3	0.00	28.86 <sup>a</sup>
ABU main campus		150	92 (61.33)	58 (38.67)				
Area BZ		16	0 (0.00)	16 (100.00)				
DAC		30	7 (23.33)	23 (76.67)				
Total		209	103 (115.43)	106 (284.57)				
Area A	<i>T. catappa</i>	8	2 (25.00)	6 (75.00)	4.06	3	0.26	38.24 <sup>a</sup>
ABU main campus		267	86 (32.21)	181 (67.79)				
Area BZ		6	4 (66.67)	2 (33.33)				
DAC		65	18 (27.69)	47 (72.31)				
Total		346	110 (151.57)	236 (248.43)				

X<sup>2</sup>: Chi-square; Df: Degree of freedom; %: Percentage; sp: species; DAC: Division of Agricultural College; ABU: Ahmadu Bello University. Mean with the same letter along the column are not significantly different at P<0.05. See sampling areas in Figure 1.

sampling sites showed that, *G. braunii* and *T. globiferus* were the most common mistletoe species found parasitic on all the targeted host

**Table 2.** Mistletoe species identified on the targeted host trees in each of the sampling areas.

Host Tree	Mistletoe species				
	Sampling areas				
	Area A	Area BZ	DAC	ABU main campus	All areas
<i>Albizzia lebbbeck</i>	<i>G. oreophila</i>	<i>T. belvisii</i>	<i>E. lecardii</i>	<i>G. oreophila</i>	<i>T. dodoneifolius</i>
	<i>G. braunii</i>	<i>T. dodoneifolius</i>	<i>T. dodoneifolius</i>	<i>T. dodoneifolius</i>	<i>T. globiferus</i> *
	<i>T. globiferus</i>	-	<i>G. oreophila</i>	<i>T. belvisii</i>	<i>G. braunii</i> *
	<i>T. dodoneifolius</i>	-	-	-	<i>G. oreophila</i>
	-	-	-	-	<i>E. lecardii</i>
	-	-	-	-	<i>T. belvisii</i>
<i>Citrus grandis</i>	<i>G. braunii</i>	<i>G. oreophila</i>	<i>T. globiferus</i>	-	<i>T. globiferus</i> *
	-	-	<i>E. lecardii</i>	-	<i>G. oreophila</i>
	-	-	-	-	<i>G. braunii</i> *
	-	-	-	-	<i>E. lecardii</i>
<i>Khaya senegalensis</i>	<i>T. globiferus</i>	<i>G. oreophila</i>	<i>G. oreophila</i>	<i>G. braunii</i>	<i>T. globiferus</i> *
	-	-	-	<i>T. globiferus</i>	<i>G. oreophila</i>
	-	-	-	-	<i>G. braunii</i> *
<i>Terminalia mantaly</i>	<i>G. oreophila</i>	-	<i>T. belvisii</i>	<i>G. braunii</i>	<i>T. globiferus</i> *
	-	-	<i>T. globiferus</i>	-	<i>T. belvisii</i>
	-	-	-	-	<i>T. dodoneifolius</i>
	-	-	-	-	<i>G. braunii</i> *
<i>Terminalia catappa</i>	<i>T. dodoneifolius</i>	<i>G. braunii</i>	<i>T. belvisii</i>	<i>T. globiferus</i>	<i>T. belvisii</i>
	<i>G. braunii</i>	-	-	<i>G. braunii</i>	<i>T. globiferus</i> *
	-	-	-	-	<i>G. oreophila</i>
	-	-	-	-	<i>G. braunii</i> *

DAC: Division of Agricultural College; ABU: Ahmadu Bello University; \* Mistletoe species found common on all the targeted host trees. *Globimetula braunii*, *Globimetula oreophila*, *Tapinanthus dodoneifolius*, *Tapinanthus belvisii*, *Englerina lecardii*, *Tapinanthus globiferus*. See sampling areas in Figure 1.

trees (*T. catappa*, *T. mantaly*, *K. senegalensis*, *A. lebbbeck* and *C. grandis*).

*G. oreophila* was found parasitic on four of the

host trees (*A. lebbbeck*, *K. senegalensis*, *T. mantaly* and *C. grandis*) and *T. belvisii* on three host trees (*A. lebbbeck*, *T. mantaly* and *T.*

*catappa*). *Englerina lecardii* was found parasitic only on two hosts (*A. lebbbeck* and *C. grandis*) and *T. dodoneifolius* was found parasitic only on *A.*

*lebbeck* (Table 2).

*A. lebbeck* had six different species of mistletoe found parasitic on it (*T. dodoneifolius*, *T. globiferus*, *G. braunii*, *G. oreophila*, *E. lecardii* and *T. belvisii*) which was the highest. *C. grandis*, *T. catappa* and *T. mantaly* each had four different species found parasitic on them whereas *K. senegalensis* had three different mistletoes species parasitic on it (Table 2).

## DISCUSSION

All the selected tree species used in this study were exotic to the region. They were planted for the provision of shade and wind break in residential areas of the region. However, the infestation of these trees by mistletoes is very high and alarming in the study area as infected trees usually have reduced vegetative growth and fruit production especially as infestation increases and are killed with time. As such, with the passage of time, if no measures are taken to curtail the rate of infestations of these trees by mistletoes, especially on *A. lebbeck*, these trees will be lost. For example, two stands of *A. lebbeck* in one of the sampling areas, Division for Agricultural College (DAC), were dead as a result of heavy infestation by mistletoes.

The tree species of area A were mostly *A. lebbeck*. This explains why it had the highest number of mistletoe species and level of infestation (although, not significantly different from those of the other tree species) as abundance of host tree in an area could influence parasitization by mistletoe as reported by (Norton and Carpenter, 1998; Norton and De Lange, 1999). Tree species like *C. grandis*, *T. catappa* and *T. mantaly* were sparsely planted in the study locations except in ABU main campus where most of it were planted. However, *C. grandis* was next to *A. lebbeck* in the level of infestation with 49.10%, although not significantly different from that of the other tree species. This result was in agreement with Asare-Bediako et al. (2013) who reported high level of infestation and very high severity indices ranging between 20 and 90% in *Citrus* trees in orchard in Ghana.

The collection of mistletoes from all the four sampling areas revealed that the host plant, *A. lebbeck* had the highest infestation (of 58.26%) of different species of mistletoes, followed by *C. grandis* and *T. mantaly* compared to the other host trees. This incidence could be attributed to the relative abundance and susceptibility of *A. lebbeck* to mistletoes more than the other host trees (except *K. senegalensis* as shown in Table 1), host choices of the mistletoes, the host plant characteristics (such as the height of the plant, branch size, susceptibility of the plant to mistletoe attack, etc) and the movement patterns of dispersal agents. Similar report was published by Aukema and Martinez (2002) and

Norton and Carpenter (1998) who reported that the relatively abundance of citrus and guava in the study area influenced the host choice of mistletoe. Overton (1994) also similarly reported that the characteristics such as branch size, age, and height of a host plant can have a strong effect on mistletoe attachment resulting in size related mistletoe infection patterns. It was also observed that, out of the several species of mistletoe obtained from these five different host trees, *T. globiferus* and *G. braunii* were the most common and found parasitic on all the five host trees. This could be due to their seeds being very sticky in nature than other mistletoe seeds, thus enhances their distribution by birds and other animals (Del Rio et al., 1996; Aukema, 2004). It can also be as a result of their being less host specific compared to the other mistletoe species (Boussim et al., 2004).

## Conclusions

These findings revealed that mistletoes could parasitize a variety of tree species with few of them having special preference to certain type of host plants. Factors like abundance of host plant and vulnerability (host characteristics) of the host plant could influence the parasitization of plant by mistletoe. Among the five studied tree species, *A. lebbeck* was the most parasitized and vulnerable to mistletoe attack in Samaru and *G. braunii* and *T. globiferus* were the most common species of mistletoe on the five host tree species.

Therefore, percentage rate of infestation of *A. lebbeck* in the study area demands the attention of authority in that region for quick measures so as to curtail the infestation, thus, preventing it from being endangered or threatened to extinction. Further studies on mistletoe species richness and the rate of infestation of other tree species within Samaru should be encouraged so as to know the presence of other species of mistletoe not identified in this study and the tree species that could be endangered or threatened by mistletoes.

## Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

## Rooting and establishment of *Limoniastrum monopetalum* (L.) Boiss stem-tip cuttings

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Rooting of *Limoniastrum monopetalum* stem-tip cuttings and establishment of produced plantlets were investigated in order to facilitate the use of the species in urban and suburban areas, and historical Mediterranean landscapes as an ornamental plant. Cuttings collected in winter or spring rooted at higher percentages than those collected in summer or autumn. Water-ethanol solutions containing 1000 to 3000 mg L<sup>-1</sup> indol-3-butyric acid (IBA) were more effective in rooting induction than the controls that did not contain IBA or powder IBA for softwood cuttings. Dipping for 1 min in an IBA solution was more effective than dipping for 5 min. Ethanol used in the IBA-solutions inhibited rooting depending on dipping time. All plantlets survived after transplantation. Plantlets transplanted on a peat-perlite (2 : 1, v/v) mixture and fertilized once a month, with 2 or 4 g L<sup>-1</sup> water-soluble complete fertilizer, had bigger elongation and produced more axillary shoots than those transplanted on a mixture amended with grape marc compost or enriched peat. Pinching of the main shoot one month after transplantation promoted axillary shoots production and a more compact plant shape.

**Key words:** *Stative monopetala* L., asexual propagation, rooting hormone indol-3-butyric acid (IBA), substrate, fertilization, ethanol rooting inhibition.

### INTRODUCTION

*Limoniastrum monopetalum* (L.) Boiss (*Stative monopetala* L., Plumbaginaceae) is a small, evergreen shrub, with much-branched, leafy stems, native in coastal sands and salt marshes in southern Greece and other Mediterranean countries (Blamey and Grey-Wilson, 1993). Due to its fleshy, silvery blue-green leaves and its

impressive bright pink, drying violet, inflorescences during summer, it is used as an ornamental plant recently. Its adaptation to a variety of environmental stresses like salinity, water deficit, intense radiation or high temperatures (Neves et al., 2008) and its growth on soil poor in organic matter content (Salama, 2007), make

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*L. monopetalum* an ideal plant for xeriscaping and landscape architecture in semi-arid Mediterranean areas, especially in poor, saline, neglected or degraded soils. Its ecological value, as sand accumulator, salt tolerant, windbreak (Salama, 2007) and inhibitor of soil erosion should not be ignored, while it can grow in oil-contaminated soils (Hussein and Terry, 2002) and has the potential of phytoremediation of heavy metals from polluted sites (Cambrollé et al., 2013; Manousaki et al., 2014).

*L. monopetalum* is rich in nutritive values and thus mass production of its vegetative yield could be raw material for fodder industries (Neves et al., 2007; Zahran and El-Amier, 2013). Moreover it is rich in phenolics, so it could constitute a source of natural antioxidants for human consumption, as well as for agro-food, cosmetic and pharmaceutical industries (Trabelsi et al., 2010, 2012, 2013).

The ability of *L. monopetalum* to grow in harsh environments along with its ornamental characteristics, led to the investigation of its asexual propagation aiming to introduce it as an ornamental plant, in urban and suburban areas and historical Mediterranean landscapes. There is no relevant information in the literature till now. The asexual propagation by stem cuttings is a simple and easily applied method of plant propagation. However, experimentation for each specific plant is necessary in order to determine the appropriate rooting hormone treatment, as well as cutting collection period. It is well established that exogenous application of auxin accelerates the rates of rooting, increases final rooting percentage and the number of produced roots in leafy cuttings (Leakey, 1990; Larson, 1992; De Klerk et al., 1999), which could be attributed to the translocation of carbohydrates and other nutrients to the rooting zone (Middleton et al., 1980; Leakey et al., 1982). However, exogenous application of auxin may be promotive, ineffective or even inhibitory for the rooting of cuttings, depending on the endogenous level of growth-regulating substances (Haissig, 1979) or the tissue sensitivity (Visser et al., 1996). Relatively high concentrations of auxins have been reported to be inhibitory to rooting, indicating that in many species, optimal concentrations for rooting have to be defined (Leakey et al., 1982). The time of collecting cuttings plays an important role in rooting success and the development of cuttings (Klein et al., 2000). This may be related to changes in the endogenous plant growth regulators or carbohydrate conditions of cuttings and the environmental conditions in nursery (Abdou et al., 2004; Elgimabi, 2008).

The aim of this study was (a) to define the appropriate season for cutting collection, (b) to determine the appropriate rooting-hormone concentration and the duration of hormone treatment (dipping time), in order to improve rooting of cuttings and (c) to test various growth mixtures and fertilizations, in order to accelerate growth of rooted cuttings, so that a complete production protocol

will be presented.

## MATERIALS AND METHODS

### Rooting of cuttings

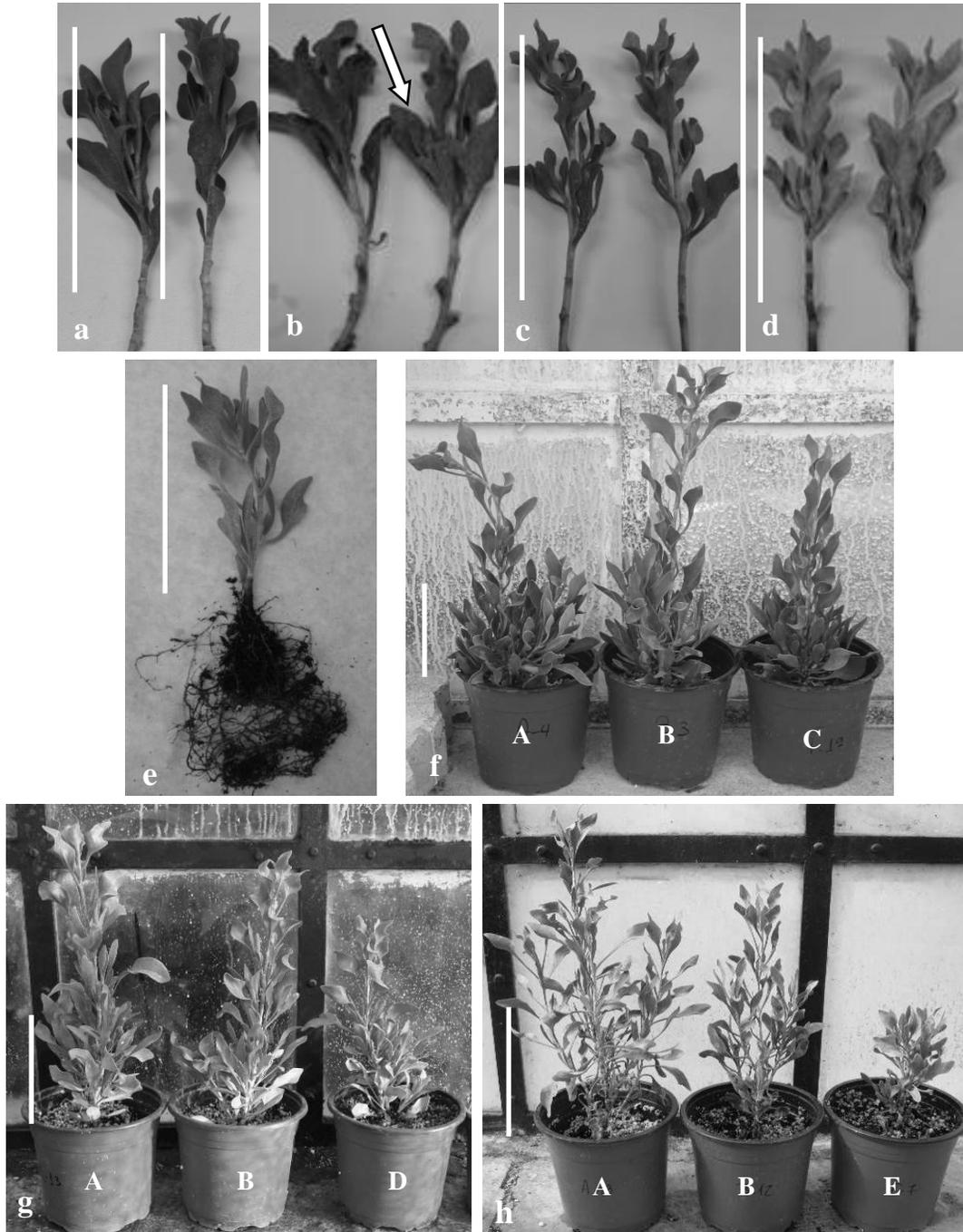
Stem-tip cuttings, 12 to 14 cm long, were excised from native *L. monopetalum* adult plants (about eight years old), grown wild in Piraeus (37°56'56.1"N, 23°38'6.5"E), in January (Figure 1a), April (Figure 1b), August (Figure 1c), and October (Figure 1d), indicative of four seasons, that is, winter, spring, summer and autumn. The experiments were carried out in two years, 2013 and 2014, but due to the similarity of the results, only data of one year are presented. In winter, cuttings were excised from the new growth, which had just sprouted. During spring, new shoots were elongated and immature inflorescences were formed at the top of some shoots, while during summer, shoot elongation was retarded and plants were in blossom. In spring and summer, cuttings were collected from non-flowering shoots. Shoot growth stopped during autumn and collected cuttings were more lignified. Generally, cuttings bear short (1.0 to 4.0 cm) axillary shoots; all leaves and axillary shoots were removed from the basal half of the cuttings (Figure 1a to d). They were treated with IBA in the form of rooting powder for herbaceous/softwood cuttings Routon DP (0.066% w/w IBA in talcum, Coordination Company of Agricultural Enterprises SA, Greece), as well as with IBA ethanol-water (1 : 1, v/v) solutions, at concentration 0 (control), 1000, 2000 or 3000 mg L<sup>-1</sup>, for two dipping times, 1 or 5 min. The bases of the cuttings were immersed (around 1.5 cm of the bottom) in the IBA solution and then placed for rooting in plastic square plug trays (cell dimensions: 5.0 × 5.0 × 5.0 cm), containing a peat (High-more with adjusted pH up to 5.5 to 6.5, Klasmann-Delimann GmbH, Geeste, Germany) and perlite (particles diameter 1 to 5 mm, Perloflor, ISOCON S.A., Athens, Greece) mixture 1 : 1 (v/v), in a mist system (spraying 15 s per 15 min from May to September or per 30 min from October to April; substrate temperature 22°C maintained by thermostatically controlled electric heating cable) for two weeks and then on a heated-glasshouse bench (37°58'53.94"N, 23°42'25.01"E) (Figure 2). Three replications with seven cuttings each were used per treatment. Rooting percentages were evaluated every two weeks for eight weeks, checking cutting resistance in pulling and root emergence through the hole at the bottom of each planting cell.

Based on initial results, where an inhibitory effect of ethanol on rooting was observed, an additional experiment was held in order to test the effect of ethanol on rooting of cuttings. Thus, the base of cuttings collected in the second half of February (end of winter) was dipped in an ethanol-water (1 : 1, v/v) solution for 1, 2.5, 5 or 10 min, as well as in plain water for 1 or 5 min (controls). Three replications with ten cuttings each were used per treatment, and rooting percentages were evaluated after eight weeks.

### Establishment of rooted cuttings

Rooted cuttings (Figure 1e) were transplanted to various mixtures in plastic pots (1.3 L), and received various fertilizations and were maintained in the glasshouse. Their growth was evaluated on a monthly basis for three months, recording the length increase of the main shoots and the number of the axillary shoots. In all experiments, three replications with seven plants each were used per treatment.

Plants produced by spring cuttings were cultured either on a peat-perlite 2 : 1 (v/v) mixture and were fertilized once a month with 2 or 4 g L<sup>-1</sup> water soluble fertilizer (Nutrileaf 60, 20-20-20, Miller Chemical and Fertilizer Corp., Hanover, PA, USA), 100 ml of solution per plant, or on a peat-perlite-grape marc compost 1 : 1 : 1



**Figure 1.** Typical stem-tip cuttings of *Limoniastrum monopetalum* collected during winter (a), spring (b), summer (c) and autumn (d), as well as 8-weeks old rooted cutting (e) (Arrow points out an axillary shoot). Typical growth of rooted cuttings collected in spring (f) and summer (g), three months after transplantation, as well as of winter cuttings, three months after pinching (h). Marked transplantation mixtures (v/v): (A) peat-perlite 2 : 1, fertilization per 30 days, 4 g L<sup>-1</sup>, (B) peat-perlite 2 : 1, fertilization per 30 days, 2 g L<sup>-1</sup>, (C) peat-perlite-grape marc compost 1 : 1 : 1, without fertilization, (D) peat-perlite-grape marc compost 3 : 2 : 1, without fertilization, and (E) enriched peat-perlite 2:1, without fertilization. Size bars = 10 cm.

(v/v) mixture, in which fertilization was not applied. Grape marc compost was produced locally, as described in Papafotiou et al. (2013), and had pH 7.8, EC 1287  $\mu\text{mhos/cm}$ , N 2.01% (by volume),

P 1.464 mg kg<sup>-1</sup>, K 15.190 mg kg<sup>-1</sup>, Mg 2.013 mg kg<sup>-1</sup> and Ca 3.667 mg kg<sup>-1</sup>. The experiment lasted from July to the end of September 2013.

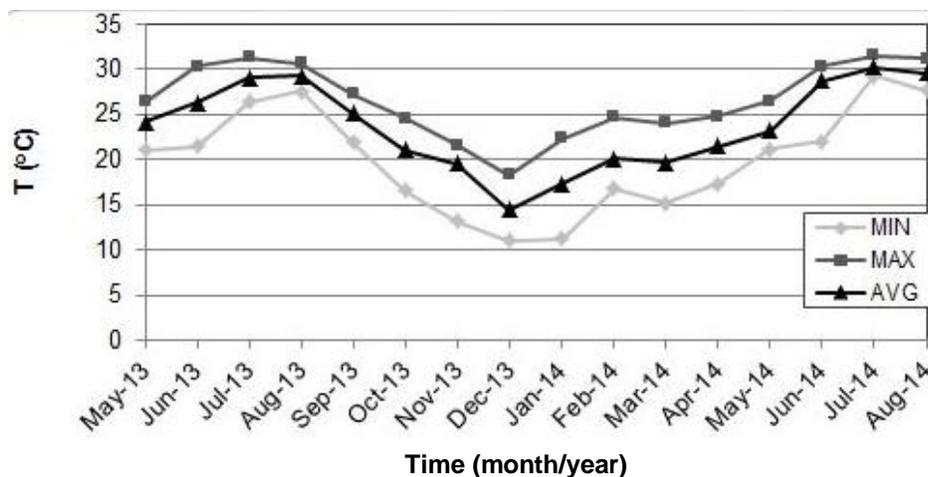


Figure 2. Monthly average temperatures (T) in the glasshouse.

Plants produced by summer cuttings were cultured either on a peat-perlite 2 : 1 (v/v) mixture and were fertilized monthly with 2 or 4 g L<sup>-1</sup> Nutrileaf 60, or on a peat-perlite-grape marc compost 3 : 2 : 1 (v/v), without fertilization. The experiment lasted from October 2013 to the end of December 2014.

Plants produced by winter cuttings were transplanted either on a peat-perlite 2 : 1 (v/v) mixture and were fertilized monthly with 2 or 4 g L<sup>-1</sup> Nutrileaf 60, or on an enriched peat (with adjusted pH up to 5.5 to 6.5, N-P-K 14-10-18 of 1.0/1.5 kg m<sup>-2</sup>, Klasmann-Delmann GmbH, Geeste, Germany) and perlite 2 : 1 (v/v) mixture, without fertilization. One month after transplantation, the main shoot of the plants was pinched (final height 10 cm) and the first fertilization was applied; the axillary shoots were not elongated during the rooting period and thus only the main shoot was pinched. One month later, data recordings started. The experiment lasted from March to the end of June 2014.

### Statistical analysis

The completely randomized design was used in all experiments. The significance of the results was tested by either one-, two- or three- way analysis of variance (ANOVA) and the means of the treatments were compared by Student's *t* test at *p* < 0.05 (JMP software, SAS Institute, Cary, NC, USA). The data on percentage were statistically analyzed after arcsine transformation. The standard error (SE) of the mean of each treatment was calculated.

## RESULTS AND DISCUSSION

### Rooting of cuttings

Three-way ANOVA of cuttings' rooting percentages showed significant interactions between season of cutting collection, IBA solution concentration and dipping time (3-way ANOVA results not presented), so rooting data were analyzed separately for each season using two-way ANOVA.

Cuttings collected in winter rooted at 100% in all treatments regardless of IBA application (Table 1). In

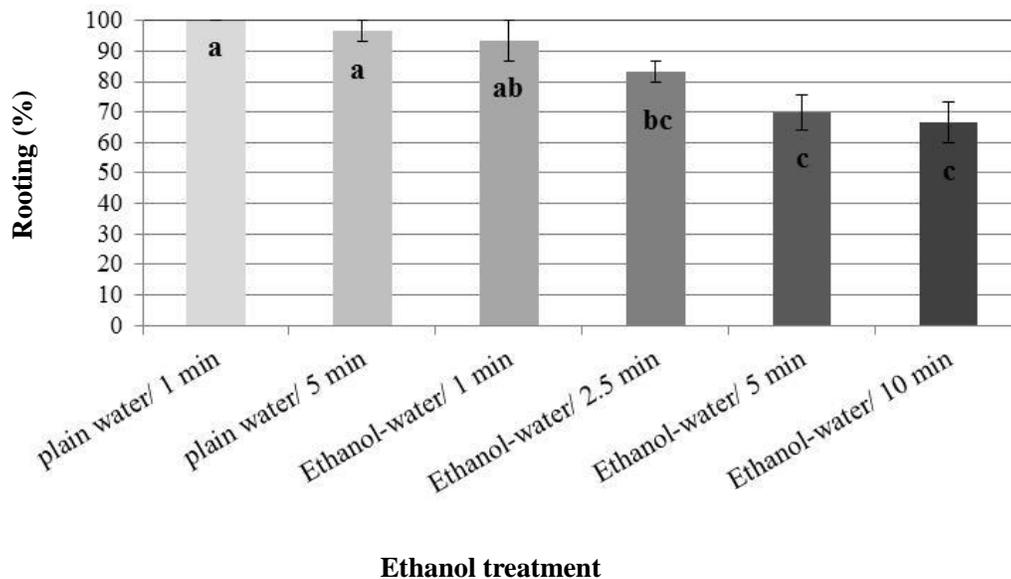
spring, there was significant interaction between the main factors of the experiment. As in winter, high rooting percentages were observed that reached 100%, with the exception of cuttings dipped for 5 min in the control or in the 3000 mg L<sup>-1</sup> IBA solution (Table 1). In summer, there was also significant interaction between the main experimental factors. Cuttings rooted at a quite high percentage after dipping their base in a 1000 or 2000 mg L<sup>-1</sup> IBA solution for 1 min, while increasing IBA concentration or dipping time reduced the response; particularly, the latter inhibited rooting, as can be seen by the comparison with the 1-min control (Table 1). In autumn, both IBA concentration and dipping time affected the response; IBA application increased rooting significantly, while longer dipping time reduced rooting. Similarly to summer, cuttings rooted at higher percentage after dipping their base in an IBA solution for 1 min compared to a 5-min dipping; maximum rooting was induced by the lower IBA concentration, but rooting was high in all three concentrations tested (Table 1). Comparison of the two controls indicates that the negative effect of 5-min dipping on rooting could be attributed to the ethanol content of the solution and not to a prolonged exposure to IBA. This indication was confirmed in the additional experiment, carried out to test a possible ethanol effect on rooting. Thus, cuttings treated with an ethanol-water solution for various dipping times rooted at higher percentage after dipping in ethanol-water solution for 1 min or the water controls as compared to those that were dipped in ethanol-water solution for longer time, 2.5, 5 or 10 min (Figure 3). Inhibition of rooting in stem cuttings by ethanol has been indicated in some previous works, as well (Middleton et al., 1978; Chong et al., 1992; De Klerk et al., 1997).

There are no reports found in the literature on propagation of the two species of *Limoniastrum* genus, *L. monopetalum* and *Limoniastrum guyonianum*; a work on

**Table 1.** Effect of collection season, IBA solution concentration ( $\text{mg L}^{-1}$ ) and dipping time (min) on rooting percentage of *Limoniastrum monopetalum* cuttings.

IBA concentration/dipping time	Season of cutting collection			
	Winter	Spring	Summer	Autumn
0 / 1	100.0 $\pm$ 0.0 <sup>a</sup>	93.4 $\pm$ 6.6 <sup>ab</sup>	35.0 $\pm$ 6.1 <sup>bc</sup>	45.0 $\pm$ 12.2 <sup>bc</sup>
1000/1	100.0 $\pm$ 0.0 <sup>a</sup>	100.0 $\pm$ 0.0 <sup>a</sup>	70.0 $\pm$ 5.0 <sup>a</sup>	100.0 $\pm$ 0.0 <sup>a</sup>
2000/1	100.0 $\pm$ 0.0 <sup>a</sup>	100.0 $\pm$ 0.0 <sup>a</sup>	70.0 $\pm$ 5.0 <sup>a</sup>	85.0 $\pm$ 6.1 <sup>ab</sup>
3000/1	100.0 $\pm$ 0.0 <sup>a</sup>	100.0 $\pm$ 0.0 <sup>a</sup>	40.0 $\pm$ 6.1 <sup>b</sup>	90.0 $\pm$ 10.0 <sup>a</sup>
0/5	100.0 $\pm$ 0.0 <sup>a</sup>	75.2 $\pm$ 6.4 <sup>b</sup>	10.0 $\pm$ 6.1 <sup>c</sup>	15.0 $\pm$ 6.1 <sup>c</sup>
1000/5	100.0 $\pm$ 0.0 <sup>a</sup>	93.4 $\pm$ 6.6 <sup>ab</sup>	10.0 $\pm$ 6.1 <sup>c</sup>	75.0 $\pm$ 7.9 <sup>ab</sup>
2000/5	100.0 $\pm$ 0.0 <sup>a</sup>	100.0 $\pm$ 0.0 <sup>a</sup>	15.0 $\pm$ 6.1 <sup>bc</sup>	50.0 $\pm$ 7.9 <sup>bc</sup>
3000/5	100.0 $\pm$ 0.0 <sup>a</sup>	75.2 $\pm$ 6.4 <sup>b</sup>	10.0 $\pm$ 6.1 <sup>c</sup>	75.0 $\pm$ 11.2 <sup>ab</sup>
$F_{\text{dipping time}}$	NS	-	-	**
$F_{\text{IBA concentration}}$	NS	-	-	**
$F_{\text{interaction}}$	NS	*	*	NS

Means  $\pm$  SE within a column followed by the same letter are not significantly different according Student's *t* test at  $p \leq 0.05$ . \* and \*\*Significant at  $p \leq 0.05$  and  $p \leq 0.01$ , respectively; NS: not significant at  $p \leq 0.05$ .

**Figure 3.** Effect of dipping time (min) in ethanol-water solutions (1:1, v/v) on rooting percentage of *Limoniastrum monopetalum* cuttings. Means  $\pm$  standard error (SE) followed by the same letter are not significantly different according Student's *t* test at  $p \leq 0.05$ .

vegetative propagation of *Plumbago capensis*, a relative plant of the Plumbaginaceae family, showed that IBA or NAA application at 1000 to 3000  $\text{mg L}^{-1}$  was necessary for rooting of subapical cuttings collected during cold period (Hernández et al., 2007), resembling the results of the present work, while hard wood cuttings of this species showed better rooting results and vegetative growth characteristics when treated with 1500 and 2000  $\text{mg L}^{-1}$  IBA (Abdulrahman and Faizy, 2013). Similarly, in the salt desert shrubs *Atriplex canescens* and *Atriplex cuneata*,

cuttings treated with various concentrations of IBA (0.1 to 2.0% in talc powder) also rooted at higher percentages than the untreated ones, particularly in seasons where rooting ability of cuttings was low (Richardson et al., 1979), as shown in the present work, too. Thus, as in most cases of leafy cuttings (Leakey et al., 1990; Larson, 1992; De Klerk et al., 1999), exogenous application of auxin promoted rooting, probably due to the translocation of carbohydrates and other nutrients to the rooting zone (Middleton et al., 1980).

**Table 2.** Effect of transplantation mixture and fertilization on growth of spring cuttings over the three months of the establishment period (July to end of September).

Time (days)	Transplantation mixture			F
	Peat-perlite 2 : 1 (v/v), fertilization monthly (2 g L <sup>-1</sup> )	Peat-perlite 2 : 1 (v/v), fertilization monthly (4 g L <sup>-1</sup> )	Peat-perlite-grape marc compost 1 : 1 : 1 (v/v), no fertilization	
<b>Main shoot length increase (cm)</b>				
30	10.0 ± 0.5 <sup>a</sup>	9.6 ± 0.5 <sup>a</sup>	9.3 ± 0.4 <sup>a</sup>	NS
60	6.0 ± 0.7 <sup>a</sup>	5.2 ± 0.5 <sup>a</sup>	2.2 ± 0.3 <sup>b</sup>	**
90	9.5 ± 0.8 <sup>a</sup>	9.1 ± 0.4 <sup>ab</sup>	7.6 ± 0.6 <sup>b</sup>	*
<b>Axillary shoot number</b>				
30	4.3 ± 0.3 <sup>a</sup>	3.9 ± 0.3 <sup>a</sup>	4.1 ± 0.3 <sup>a</sup>	NS
60	5.3 ± 0.2 <sup>a</sup>	4.2 ± 0.3 <sup>b</sup>	4.3 ± 0.3 <sup>b</sup>	*
90	6.0 ± 0.2 <sup>a</sup>	4.7 ± 0.3 <sup>b</sup>	4.7 ± 0.3 <sup>b</sup>	*

Means ± standard error (SE) within a line followed by the same letter are not significantly different according Student's t test at  $p \leq 0.05$ . \* and \*\*Significant at  $p \leq 0.05$  and  $p \leq 0.01$ , respectively; NS: not significant at  $p \leq 0.05$ .

The superiority of winter may be due to the fact that new vegetation of *L. monopetalum* sprouts in the middle of winter, and cuttings collected during this period, may be richer in endogenous auxins produced at the active apex of the young shoot and transported basipetally to the base of the cutting acting as a trigger for rooting (Nordström and Eliasson, 1991). Higher rooting percentages of cuttings during growing season has been reported for other salt desert shrubs, such as *A. canescens* and *A. cuneata*, too (Richardson et al., 1979), while the opposite was shown for *Artemisia tridentata*, plant that can grow in very alkaline and dry soils, where peak rooting percentages of cuttings were produced in late winter and root formation was much reduced after the onset of growth in spring (Alvarez-Cordero, 1979). Ambient temperature is rather unlikely to have affected rooting, as temperature in the mist was quite constant.

Auxin concentrations exceeding a certain level have been reported to inhibit or reduce rooting ability of stem cuttings of various species (Leakey et al., 1982; Chong et al., 1992; Puri and Verma, 1996; Akwatulira et al., 2011), indicating that optimal concentrations for rooting have to be defined (Leakey et al., 1982; Chong et al., 1992).

Cuttings collected from spring to autumn and treated with powder IBA for soft-wood cuttings rooted at lower percentages (5 to 45%), compared to those that were dipped in 0 or 1000 mg L<sup>-1</sup> IBA solution (the latter contains similar quantity of rooting hormone to powder) for 1 min. Only those collected in winter, during which cuttings generally rooted easily at high percentages irrespectively of treatment, rooted at 90% (data not shown). These results are consistent with those obtained in various landscape shrubs and trees, where talc-IBA formulations were less effective than IBA in solution at comparable concentrations in rooting of stem cuttings (Chong et al., 1992).

Regarding the required time for rooting, cuttings collected in winter and spring and dipped in solutions 1000 to 3000 mg L<sup>-1</sup> IBA for 1 min (the best treatments throughout the year) rooted faster, reaching the maximum of their rooting percentage at only two weeks, compared to cuttings collected in summer or autumn, which reached their maximum rooting percentage after 6 to 8 weeks (data not shown).

### Establishment of rooted cuttings

Establishment of rooted cuttings was successful and all plantlets survived three months after transplantation independently of substrate and fertilization type, season and culture technique applied. Plantlets that were fertilized monthly with a water soluble fertilizer exhibited bigger elongation of the main shoot and, in general, produced more axillary shoots compared to those that were not fertigated, but instead their substrate was amended with grape marc compost or enriched peat (Tables 2 to 4 and Figure 1f to h). Grape marc compost is of high quality, and it is degraded very slowly providing good physical structure to amended mixture and releasing slowly its nutrients (Manios, 2004). Thus, plantlets cultured in the compost amended mixture probably had less nitrogen available to promote shoot elongation compared to those that were fertigated, particularly during periods with lower temperatures (November to January, Figure 2), when nitrogen release from compost was probably lower compared to periods with high temperatures (Agehara and Warncke, 2004). Similarly enriched peat provided much fewer nutrients to the plants compared to monthly fertilization (see materials and methods), resulting in the smallest shoot elongation (Table 4 and Figure 1h). During the hottest

**Table 3.** Effect of transplantation mixture and fertilization on growth of summer cuttings over the three months of the establishment period (October to end of December).

Time (days)	Transplantation mixture			F
	Peat-perlite 2 : 1 (v/v), fertilization monthly (2 g l <sup>-1</sup> )	Peat-perlite 2 : 1 (v/v), fertilization monthly (4 g l <sup>-1</sup> )	Peat-perlite-grape marc compost 3 : 2 : 1 (v/v), no fertilization	
<b>Main shoot length increase (cm)</b>				
30	4.6 ± 0.2 <sup>b</sup>	7.3 ± 0.3 <sup>a</sup>	4.0 ± 0.2 <sup>b</sup>	**
60	4.3 ± 0.2 <sup>b</sup>	5.3 ± 0.3 <sup>a</sup>	1.6 ± 0.2 <sup>c</sup>	**
90	2.7 ± 0.1 <sup>a</sup>	3.1 ± 0.2 <sup>a</sup>	1.3 ± 0.1 <sup>b</sup>	**
<b>Axillary shoot number</b>				
30	4.2 ± 0.1 <sup>a</sup>	4.5 ± 0.1 <sup>a</sup>	3.6 ± 0.1 <sup>b</sup>	**
60	5.9 ± 0.1 <sup>a</sup>	6.2 ± 0.2 <sup>a</sup>	4.3 ± 0.3 <sup>b</sup>	**
90	9.4 ± 0.4 <sup>a</sup>	9.0 ± 0.3 <sup>a</sup>	7.7 ± 0.3 <sup>b</sup>	**

Means ± standard error (SE) within a line followed by the same letter are not significantly different according Student's t test at  $p \leq 0.05$ . \*\*Significant at  $p \leq 0.01$ .

**Table 4.** Effect of transplantation mixture and fertilization on growth of winter cuttings pinched in April, one month after transplantation, over three months (April to end of June); fertilizations started after pinching.

Time (days)	Transplantation mixture			F
	Peat-perlite 2 : 1 (v/v), fertilization monthly (2 g L <sup>-1</sup> )	Peat-perlite 2 : 1 (v/v), fertilization monthly (4 g L <sup>-1</sup> )	Enriched peat-perlite 2 : 1 (v/v), no fertilization	
<b>Length of main shoots (cm)</b>				
30	6.4 ± 0.2 <sup>b</sup>	9.0 ± 0.3 <sup>a</sup>	5.0 ± 0.2 <sup>c</sup>	**
60	9.0 ± 0.2 <sup>b</sup>	12.4 ± 0.5 <sup>a</sup>	5.4 ± 0.2 <sup>c</sup>	**
90	11.4 ± 0.3 <sup>b</sup>	16.3 ± 0.6 <sup>a</sup>	5.7 ± 0.2 <sup>c</sup>	**
<b>Main shoot number</b>				
30	6.4 ± 0.2 <sup>a</sup>	6.1 ± 0.3 <sup>ab</sup>	5.5 ± 0.2 <sup>b</sup>	*
60	6.8 ± 0.2 <sup>a</sup>	7.0 ± 0.3 <sup>a</sup>	5.6 ± 0.2 <sup>b</sup>	**
90	6.9 ± 0.2 <sup>a</sup>	7.2 ± 0.3 <sup>a</sup>	5.7 ± 0.2 <sup>b</sup>	**

Means ± standard error (SE) within a line followed by the same letter are not significantly different according Student's t test at  $p \leq 0.05$ . \* and \*\*: significant at  $p \leq 0.05$  and  $p \leq 0.01$ , respectively.

months of the year, July to August, (Figure 2), the dose of fertilizer applied did not seem to affect shoot elongation (Table 2), while during cooler periods (October to December and March to June) shoot elongation was promoted by the bigger fertilizer dose (Tables 3 and 4). Plantlets growth was characterized by strong apical dominance; thus, plantlets produced by spring or summer cuttings, which were not pinched, developed only one main shoot, bearing some axillary shoots, which were not elongated (Figure 1f). Apical dominance determines the degree of branching and the form of the shoot system (Cline, 1994; Leyser, 2003). Auxin, derived from the shoot apex and moving basipetally, inhibits growth of axillary buds, while cytokinin, thought to be derived from

the roots, promotes their growth (Cline, 1994; Leyser, 2003). Tanaka et al. (2006) indicated that one role of auxin is to repress local biosynthesis of cytokinins in the nodal stem and that, after decapitation, cytokinins are locally biosynthesized in the nodal stem rather than in the roots. The reduced main shoot elongation during winter (December) probably restricted apical dominance and thus more axillary buds were developed at this period (Table 3), resembling the species behaviour in the wild, where new vegetation sprouts in winter.

Plantlets produced by winter cuttings were established during spring and had their main shoot pinched one month after transplantation in order to remove apical dominance. As expected, axillary shoots were elongated

and so more main shoots per plant were formed three months later; produced main shoots had no axillary shoots on (Table 4 and Figure 1h). In this way, a rounded and thus more attractive plant shape was taken, particularly when plants were fertigated with the higher fertilizer dose (Figure 1h).

In general, plantlets transplanted on a mixture of peat and perlite and received fertigation were more vigorous than those transplanted on a mixture containing grape marc compost or enriched peat and were not fertilized. The higher the dose of fertilizer used, the taller the plants became, which is not always desirable because plants may bend easily or develop less attractive shape if not pinched. During the experiments, it was observed that plants fertilized with the higher dose were also less tolerant to water deficiency.

## Conclusion

A complete production protocol of *L. monopetalum* is provided. Rooting of cuttings was affected both by season and rooting hormone treatment. Winter and spring were the most appropriate collection seasons and dipping in 1000 or 2000 mg L<sup>-1</sup> IBA for 1 min was the best treatment. As regards establishment, growth of rooted cuttings was enhanced by transplantation on a peat-perlite mixture (2:1, v/v) and monthly fertigation. Pinching was necessary for the production of branched plants.

## Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

# Effects of soil substrate quantity and sowing method on cocoa (*Theobroma cacao* L.) seedlings growth in Togo

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Cocoa is one of the outstanding cash crops in Togo, but the orchards are old and require the rehabilitation through replanting and good cocoa seedlings. A study was conducted in nursery to investigate the effect of soil quantity and sowing method on cocoa seedlings growth. The treatments were a quantity of depleted bulk soil in two pots (2120 and 1055 cm<sup>3</sup>) and the cocoa seeds were sown by three methods: Direct sowing in pot and replanting small seedling growth after 10 and 15 days on seedbed. The seedlings were regularly watered in the same conditions and data were collected on growth parameters (seed germination ratio or seedlings lifting percentage, root length, stem girth, plant height and number of leaves). As demonstrated by the results, the growth parameters of cocoa seedlings in the two quantities of soil are not significantly different ( $p < 0.05$ ). Therefore, no difference is found between the effects of soil quantity on seedlings growth, while it is affected by sowing method. Replanting cocoa small seedlings must be done 10 days after seed lifting. It is economic for farmers to use the small pots (1055 cm<sup>3</sup>) for cocoa nursery instead of the big one as recommended actually.

**Key words:** *Theobroma cacao* L., soil quantity, direct sowing, replanting.

## INTRODUCTION

Cocoa (*Theobroma cacao* L.) is one of the outstanding cash crops in Togo. Cocoa contributes 1.3% to the Gross Domestic Product of Togo in 2010 (l'Institut National de la

Statistique et des Etudes Economiques et Démographiques [INSEED], 2015). Togolese cocoa orchards were estimated at 23,290 ha, most of which

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were declining: 40% of cocoa farms have 5 to 20 years old and 32% older than 20 years old. The cocoa network occupies 17% of agriculture active labour in Togo. An annual estimated cocoa beans production is around 6000 Mg with an average yield of 242 kg.ha<sup>-1</sup> (Direction de la Statistique, de l'Informatique et de la Documentation [DSID], 2014). Therefore, there is need to intensify its production by yield improvement through rehabilitation of old and non-producing cocoa plantations and by renewing the orchard through increased land area cultivated or new plantation establishment (Oyewole et al., 2012; Ogunlade, 2008).

In an attempt to increase the cocoa farms yields with the target of increasing sustainable supply to the world market, farmers are encouraged to replant their old cocoa farms and this is managed under the project called "Projet d'Appui au Secteur Agricole (PASA)" funded by World Bank with counterpart contribution from Togolese Government. In this context, farmers need good cocoa seedlings for replanting their plantations. Unfortunately, cocoa seedlings growth faces a lot of challenges in cocoa production lands in Togo such as cocoa pot transportation from nursery to orchard disagreements, because of pots heaviness and cocoa beans losses due to relative late of seeds sowing in pot already filled. Over the past few years, farmers have developed an unusual approach to grow cocoa seedlings by using coffee pot (21 × 8 cm<sup>2</sup>) instead of cocoa one (27 × 10 cm<sup>2</sup>) to reduce soil volume then pot heaviness. They sow cocoa seeds on seedbed before replanting it in pot when they are free.

Several authors worked on cocoa seedlings growth conditions in nursery. Harun and Ismail (1983) worked on cocoa seedlings shading regimes while many researchers reported results on soil quality, not too much on soil quantity. Publishers communicated enough results on soil texture, soil chemical composition, soil supply of nutrients and soil nutrients requests for cocoa seedlings or tree production, mostly on soil supply of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg). Furthermore, the direct sowing of cocoa beans in pot was almost suggested as a sowing method (Akanbi et al., 2014; Koko, 2014; Oyewole et al., 2012; Koko et al., 2009; Mohd.Yusoff et al., 2007; Hartemink, 2005).

With the works of Jardin and Snoeck (1985), Snoeck and Jardin (1992) and Snoeck et al. (2006), nutrient requirements for cocoa tree growth can be computed by Soil-Diagnostic Method. In this approach, soil quality and soil quantity is required to evaluate the real needs for cocoa trees. So in nursery, the seedling growth depends on the available nutrients containing in a specific soil volume in the pot. Although, soil quality is found to have an effective effect on cocoa tree growth (Akanbi et al., 2014; Koko, 2014; Koudjega and Tossah, 2009; Mohd.Yusoff et al., 2007), its quantity influence on growth and performance of cocoa seedlings have not yet

received adequate research attention as well as sowing method in nursery in Togo conditions.

Therefore, the objectives of this study were to determine the effect of soil quantities on cocoa seedlings growth and to evaluate the influence of different sowing methods on cocoa seedlings growth.

## MATERIALS AND METHODS

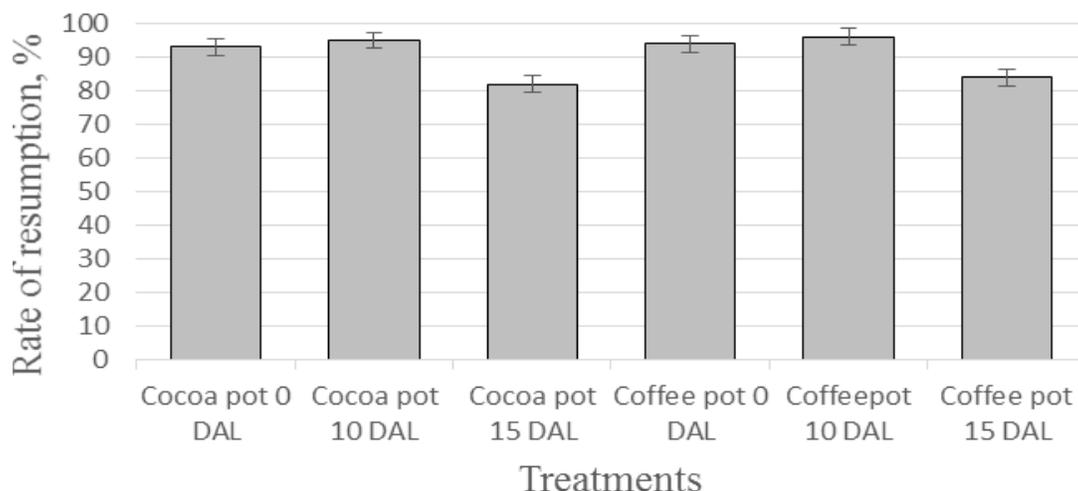
The experiment in nursery was conducted for five months and half in the Vegetal Material Production Center of Ezimé in Togo. This center is linked to Unité Technique Café Cacao (UTCC, a Cocoa and Coffee Extension Service in Togo). It is located between latitude 07°29'31" N and longitude 0°56'83" E lying on an altitude 252 m above sea level, 4 km from Amlamé town, along National Road N°5.

A Randomized Complete Block Design with four replications and two factors, soil quantities and sowing methods, was used for the control of spatial variability (van Es and van Es, 1993; van Es et al., 2004). Each plot consisted of 25 pots separated by 20 cm from each other and one cocoa bean or one cocoa small seedling was directly sown or replanted into each of the pot. Watering was done regularly later in the evening to field capacity every two days interval.

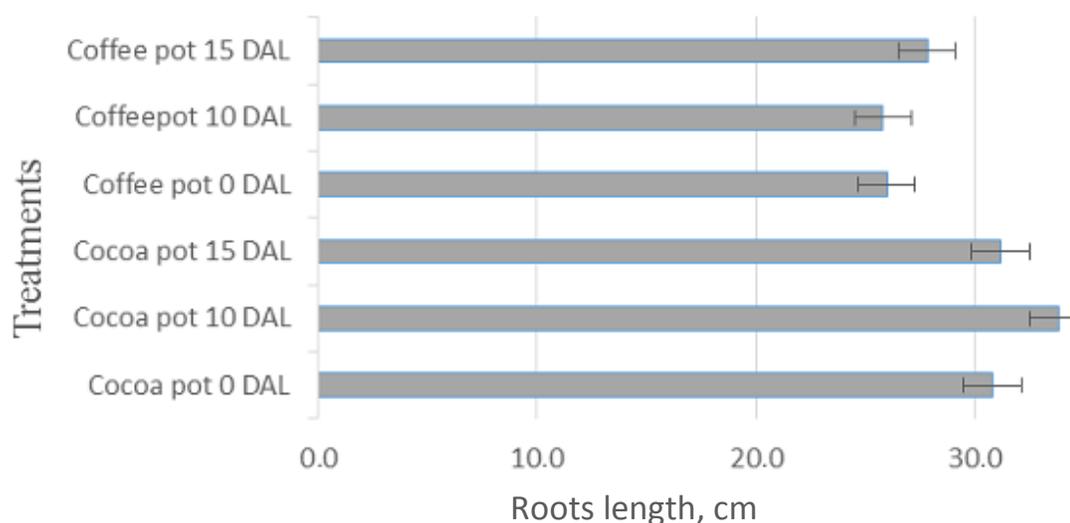
The plastic pots used were the ordinary polyester black plastic bag with dimensions of 21 × 8 cm, called "coffee pots" (1055 cm<sup>3</sup>) and 27 × 10 cm called "cocoa pots" (2120 cm<sup>3</sup>). The cocoa seeds were gotten from Ghana Cocoa Board and a hybrid varieties seeds obtained by crossing and mixing the following genetic materials: 77 × 42 (33%), 77 × 85 (34%), and 77 × 67 (33%). Depleted soil (0 to 20 cm) from the center was collected at the beginning of the trial, air dried, crushed and sieved handily to serve as potting media. This soil is described as a silty clay, rich in organic matter (55 g.kg<sup>-1</sup>), poor in nitrogen (2 g.kg<sup>-1</sup>) and phosphorus (21.9 mg.kg<sup>-1</sup>). Exchangeable bases levels were low (0.3 K, 13.4 Ca and 5.9 Mg meq%) with cation exchange capacity (CEC) equal to 21.1 meq% and the pH was 6.7 (Koudjega and Tossah, 2009). Bulk soil was properly mixed to ensure homogenous soil and filled into each perforated plastic pot of different capacity leaving some space for watering and then placed under shade with dimensions 4 × 3 × 2 m<sup>3</sup>, controlled to prevent the passage of 80% of light (Mohd.Yusoff et al., 2007). The seeds were sown on December 8th, 2014 by three methods: direct sowing in pot (0 day after lifting, DAL) and replanting small seedlings growth after 10 days on seedbed, then after 15 days on seedbed (10 and 15 DAL).

The following growth measurements were taken at two months interval during the experiment from January 15th, 2015 (1.5 Months After Planting, MAP) up to May 18th, 2015 (5.5 MAP): seed germination ratio or small seedling lifting percentage, root length, stem girth, plant height and number of leaves. The seed germination ratio is calculated by dividing the number of seedlings alive two weeks after sowing over the number of seed sown in the pot in each treatment multiplied by hundred. The seedlings lifting percentage is calculated by dividing the number of seedlings alive one month after replanting over the number of small seedlings replanted in the pot in each treatment multiplied by hundred. For root length measurement, three seedlings in pot in each treatment were sacrificed, then soil in the pot was removed and the root was measured on graduated paper. The root length in each treatment is the average of the three length measured on the three seedlings roots. For stem girth size, the diameter at the base of all seedlings in each treatment was measured, then the averaged circumference of the seedling base was calculated. The plant height was measured from the seedlings base up to the plant summit.

Analysis of variance (ANOVA) and Duncan Multiple Range Test



**Figure 1.** Resumption rates as affected by soil substrate quantities and sowing method.



**Figure 2.** Roots length as affected by soil substrate quantities and sowing methods.

(DMR) were used on a statistical software STATISTICA version 5.5 (StatSoftInc, 1999) at 5%.

## RESULTS

The cocoa seedlings lifted very well in almost all the treatments during the trial (Figure 1). The best resumption or germination ratio appeared at 10 DAL in both cocoa and coffee pots, and averaged, respectively at  $95\pm 3.8$  and  $96\pm 3.3\%$ . There were no significant effects of the two types of pots on seedling lifting ( $90\pm 7\%$  for cocoa pots and  $91\pm 6\%$  for coffee pots), while the sowing methods revealed a negative impact of later replanting of

small cocoa seedlings. With replanting 15 DAL, cocoa seedlings lifted averagely at  $84\pm 9.2\%$  in coffee pots and  $82\pm 12\%$  in cocoa pots.

No significant difference was found in the cocoa seedlings roots length across all treatments ( $F_{(5,24)}=0.29$ ;  $p=0.91$ ). The root length was the same ( $29.3\pm 3.2$  cm) in the two soil quantities and across the three sowing methods. However, it was noticed that all roots from cocoa pots were beyond 30 cm and those from coffee pots were less than 30 cm (Figure 2) with no statistical difference between them.

The stem girth of cocoa seedlings (Table 1) in both cocoa and coffee pots were significantly influenced by sowing methods during the trial. At 1.5 MAP, the direct

**Table 1.** Effects of soil quantity and sowing methods on cocoa seedlings stem girth (cm).

Soil quantity	Sowing method	1.5MAP		3.5MAP		5.5MAP	
		Means	SD	Means	SD	Means	SD
1055 cm <sup>3</sup> (Coffee pot)	0 DAL	1.27 <sup>a</sup>	0.05	1.35 <sup>d</sup>	0.01	1.75 <sup>bc</sup>	0.21
	10 DAL	1.02 <sup>b</sup>	0.07	1.67 <sup>b</sup>	0.02	2.00 <sup>a</sup>	0.10
	15 DAL	0.93 <sup>b</sup>	0.05	1.80 <sup>a</sup>	0.01	1.92 <sup>ab</sup>	0.18
2021 cm <sup>3</sup> (Cocoa pot)	0 DAL	1.27 <sup>a</sup>	0.05	1.35 <sup>d</sup>	0.07	1.58 <sup>cd</sup>	0.18
	10 DAL	1.02 <sup>b</sup>	0.07	1.47 <sup>c</sup>	0.09	1.40 <sup>d</sup>	0.09
	15 DAL	1.02 <sup>b</sup>	0.07	1.64 <sup>b</sup>	0.07	1.48 <sup>d</sup>	0.05
Means	-	1.09	0.06	1.55	0.04	1.67	0.14
F	-	18.35		55.20		10.95	
p-level	-	0.00		0.00		0.00	

DAL: Day after lifting; MAP: months after planting. SD: standard deviation. Means in the same column denoted by same letters are not significantly different with ANOVA and DMR at 5%.

**Table 2.** Effects of soil quantity and sowing methods on cocoa seedlings height (cm).

Soil quantity	Sowing method	1.5MAP		3.5MAP		5.5MAP	
		Means	SD	Means	SD	Means	SD
1055 cm <sup>3</sup> (Coffee pot)	0 DAL	13.25 <sup>a</sup>	0.64	23.38 <sup>a</sup>	4.72	30.50 <sup>a</sup>	2.40
	10 DAL	13.23 <sup>a</sup>	1.25	19.75 <sup>a</sup>	2.54	26.92 <sup>ab</sup>	3.25
	15 DAL	13.65 <sup>a</sup>	0.76	23.80 <sup>a</sup>	0.90	23.33 <sup>b</sup>	3.93
2120 cm <sup>3</sup> (Cocoa pot)	0 DAL	13.68 <sup>a</sup>	0.92	22.27 <sup>a</sup>	2.41	26.15 <sup>ab</sup>	3.26
	10 DAL	13.95 <sup>a</sup>	0.27	19.73 <sup>a</sup>	1.44	30.25 <sup>a</sup>	3.33
	15 DAL	13.38 <sup>a</sup>	1.77	23.73 <sup>a</sup>	4.72	19.10 <sup>c</sup>	1.24
Means	-	13.52	0.93	22.11	2.79	26.04	2.90
F	-	0.29		1.85		10.01	
p-level	-	0.91		0.15		0.00	

DAL: Day after lifting; MAP: months after planting; SD: standard deviation. Means in the same column denoted by same letters are not significantly different with ANOVA and DMR at 5%.

sowing (0 DAL) generated the best seedlings stem girth average with  $1.3 \pm 0.1$  cm, then 15 DAL in coffee pots showed the best seedlings stem girth at 3.5 MAP ( $1.8 \pm 0$  cm) and finally at 5.5 MAP, treatments with replanted small cocoa seedlings (10 and 15 DAL) in coffee pots revealed the best cocoa seedlings stem girth with  $2.0 \pm 0.1$  and  $1.9 \pm 0.2$  cm, respectively.

The tallest plants at 5.5 MAP were produced by both direct sowing in coffee pots and replanted small cocoa seedlings at 10 DAL in cocoa pots ( $30.5 \pm 2.4$  and  $30.3 \pm 3.3$  cm, respectively). At the same stage, plants in coffee pots were averagely taller than those in cocoa pots with  $26.9 \pm 3.6$  and  $25.2 \pm 5.6$  cm, respectively (Table 2). Statistically, no difference was found in plant height before 5.5 MAP.

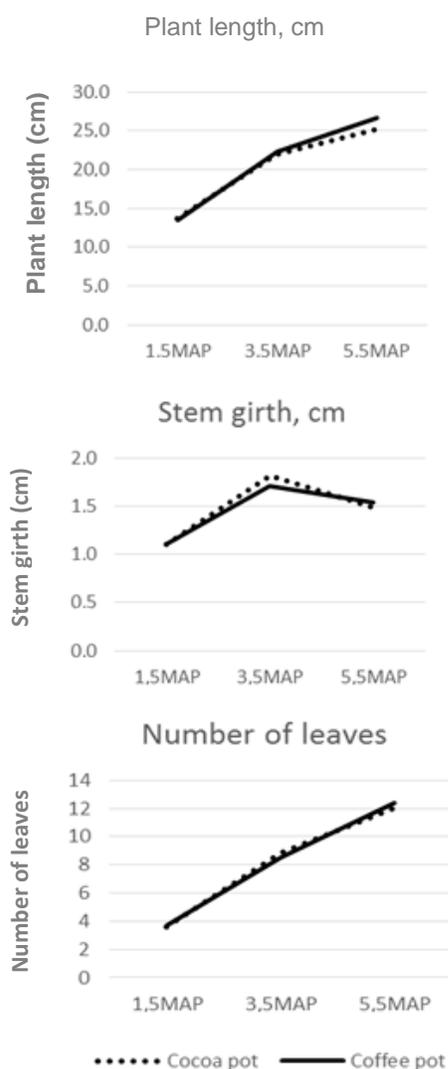
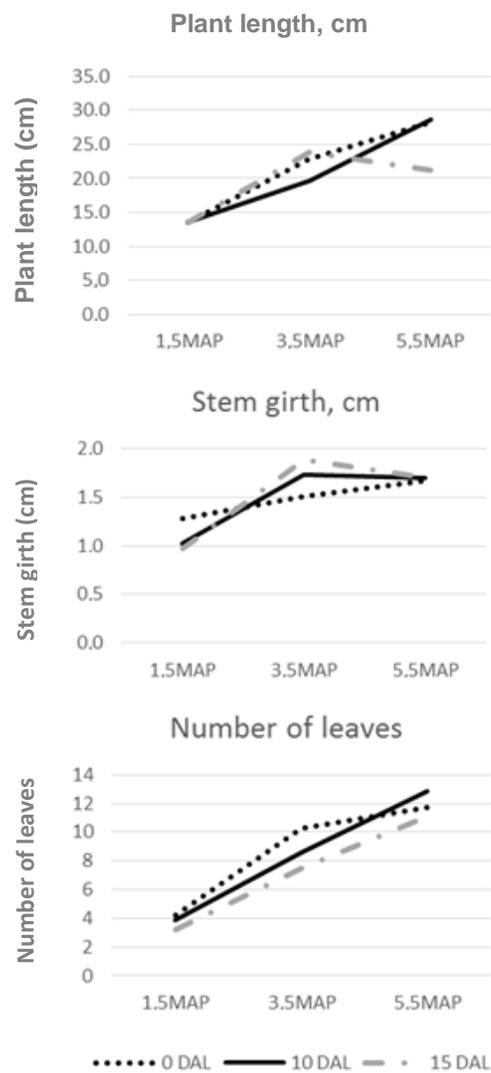
There was no significant difference in the leaves number at the final stage and averaged at  $12 \pm 1$  leaves on cocoa seedlings (Table 3). Soil quantities and sowing methods had no effect on cocoa seedlings leaves production at 5.5 MAP.

Soil quantities seem to have no influence on cocoa seedlings performance (Figure 3) while sowing methods have impacted the plant length, stem girth and leaves number (Figure 4). A weak positive correlation was found, at 5.5 MAP, between plant height and seedlings resumption, then plant height and leaves number finally between plant height and stem girth size ( $0.03 \leq R^2 \leq 0.56$ ). A strong positive correlation is noted between leaves number and stem girth size ( $0.47 \leq R^2 \leq 0.70$ ). So, as quick as the cocoa seedlings are lifted, more stem girth is

**Table 3.** Effects of soil quantity and sowing methods on cocoa seedlings leaf number.

Soil quantity	Sowing method	1.5 MAP		3.5 MAP		5.5 MAP	
		Means	SD	Means	SD	Means	SD
1055 cm <sup>3</sup> (Coffee pot)	0 DAL	5 <sup>a</sup>	0.6	10 <sup>a</sup>	2.2	12 <sup>a</sup>	1.1
	10 DAL	4 <sup>abc</sup>	0.5	9 <sup>ab</sup>	0.6	12 <sup>a</sup>	1.9
	15 DAL	3 <sup>c</sup>	0.5	7 <sup>c</sup>	0.6	11 <sup>a</sup>	2.6
2120 cm <sup>3</sup> (Cocoa pot)	0 DAL	4 <sup>abc</sup>	0.0	11 <sup>a</sup>	0.6	11 <sup>a</sup>	2.0
	10 DAL	4 <sup>abc</sup>	0.6	8 <sup>bc</sup>	0.7	14 <sup>a</sup>	1.6
	15 DAL	3 <sup>c</sup>	0.5	8 <sup>bc</sup>	0.4	11 <sup>a</sup>	1.3
Means	-	4	0.5	9	1.3	12	1.0
F	-	4.84		6.60		1.53	
p-level	-	0.01		0.00		0.23	

DAL: Day after lifting; MAP: months after planting; SD: standard deviation. Means in the same column denoted by same letters are not significantly different with ANOVA and DMR at 5%.

**Figure 3.** Cocoa seedlings growth as affected by soil quantities.**Figure 4.** Cocoa seedlings growth as affected by sowing methods.

raised, more plant grown than more leaves appeared.

## DISCUSSION

The lifting percentage of the small seedlings or germination ratio of cocoa seeds, in both coffee and cocoa pots, was superior to 90% as recommended by Togolese seeds norms, except in the case of replanting small seedling 15 DAL where the ratio is still good ( $\geq 80\%$ ) according to West African countries seeds production norms. The cocoa seedling root length after 4 to 5 months growth must be around 30 to 40 cm according to Oro (2011) and LooorSolorzano (2007). Results from the current study ( $29.3 \pm 3.2$  cm) seem to be similar to the previous results. Oyewole et al. (2012) worked on cocoa seedlings in Nigeria and published at 6 MAP a stem girth that ranged from 1.9 to 3.1 cm and plant height between 17 and 59 cm. Bismark (2011) published that, in Ghana, the cocoa seedling height must be 25 to 43 cm at 5 MAP, while leaves number must be 8 to 18. These finds are in agreement with the results of the current study where the cocoa seedling stem girth is  $1.4 \pm 0.1$  to  $2.0 \pm 0.1$  cm and cocoa seedling height is  $19.1 \pm 1.2$  to  $30.5 \pm 2.4$  cm, while leaves number is 11 to 14. So, the agronomic performance of cocoa seedlings obtained in this trial is in agreement with those obtained in Nigeria and Ghana. No significant difference was found between the cocoa pots and the coffee pots on cocoa seedling growth confirmed by Bismark (2011) who also found no difference in water sachets, Cocobod poly bags and International Institute of Tropical Agriculture (IITA) poly bags that are used to grow cocoa seedlings in Ghana. The positive correlation found between cocoa seedling growth variables explains the relationship between these variables. More cocoa seedling upsurges more stem girth increases, more leaves number rises.

Actually, to produce cocoa seedlings for 1 ha, farmers need 1800 plastic pots as recommended by extension services. Cocoa pot cost on the market is 15 FCFA, whereas coffee pot price is 8 FCFA. Therefore, farmers can reduce their charge by 7 FCFA per pot. This represents an important benefit of 12600 FCFA per hectare for nursery preparation for small holders' farmers in cocoa production lands in Togo without counting the easiness of coffee pots filling and its carrying from nursery to cultivated area.

## Conclusion

The growth and performances of cocoa seedlings nursed in different pots seem to be the same in all treatments according to the plants growth variables measured and statistically tested. The two soil quantities had no effects on cocoa seedlings growth and no influence of the three sowing methods was found on cocoa seedlings growth except the replanting 15 DAL. Therefore, the use of

"coffee pots" ( $21 \times 8$  cm) for cocoa seedlings production instead of the cocoa pots ( $27 \times 10$  cm), as recommended actually, is economic for farmers who can reduce their charge in nursery. As an alternative for cocoa seeds direct sowing, replanting cocoa small seedlings growth on seedbed must be done at the latest 10 days after seeds are raised. If soil quantities have no significant influence on cocoa seedlings, it will be interesting to know how the plant nutrients uptake and nutritional pool will be in these specific soil quantities in the pot.

## Conflict of Interests

The authors have not declared any conflict of interest.

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## Short Communication

## ***Iphimeis dives* (Chrysomelidae) Beetle occurrence in beans in western Parana State, Brazil**

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***Iphimeis dives* (Germar, 1824) is a species of beetle from Chrysomelidae family, commonly found in fruit trees, and it is known as a vine defoliator beetle or Green Beetle. In this study, *I. dives* occurrence in bean (*Phaseolus vulgaris* L.) is registered in the cities of Assis Chateaubriand and Palotina, western region of Paraná State. This is the first recorded *I. dives* attack in Brazil's bean crops. The insects were collected when plants were between the phenological development stages V4/R5. Although commonly found in fruit trees and some vegetables, this record in bean crop suggests greater attention to this insect because of the damage it may cause in this culture.**

**Key words:** *Phaseolus vulgaris* L., defoliator beetle, green beetle.

### INTRODUCTION

Beans (*Phaseolus vulgaris* L.) is the Brazilian population's staple food, being an excellent food since it provides essential nutrients to humans such as protein, iron, calcium, magnesium, zinc, vitamins, carbohydrates and fiber (Mesquita et al., 2007). In addition, bean has a great socio-economic importance as it is grown by small, medium and large producers throughout the country, in diverse production systems (Moura and Brito, 2014). The total grain production in Brazil's 2013/14 crop was 3.45 million tons in an area of 3.36 million hectares, whereas Paraná state had a production of 808,900 tons of grains (Conab, 2015), being the largest domestic producer. However, the crop can be affected by various insects, including Chrysomelidae family coleopterous (Oliveira and Ramos, 2012), especially

*Diabrotica speciosa* and *Cerotoma arcuata*, known as little cows (Moura et al., 2014; Pratisoli et al., 2012; Schmildt et al., 2010). In adulthood, these insects reduce the plants leaf area (Quintela and Barbosa, 2014) and it may cause significant decrease in the photosynthetic capacity of the crop, resulting in decreased productivity (Pratisoli et al., 2012; Schmildt et al., 2010; Silva et al., 2003).

The Chrysomelidae family consists of various coleopterous, including the *Iphimeis dives* species (Germar, 1824), also known as vine defoliator beetle or green beetle, which has been reported attacking various fruit trees, some crops and vegetables (Basso et al., 1974; Mariconi, 1962; Milléo et al., 2013; Wiest and Barreto, 2012). According to Quintela and Barbosa

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(2014), coleopterous of this family in adulthood cause defoliation throughout the bean crop cycle, and they may feed on flowers and pods. The most significant damage occurs in the seedling stage, since they may consume the apical bud. In case of a high insect population, there might not be available leaf area, causing plant death.

Silva et al. (2003) point out that the bean's ability to recover after defoliation varies according to the development stage it is subject to damage. Glier et al. (2015) observed that defoliation in V4 and R5 stages are the most harmful to the crop, occurring greater reduction in production potential. Similarly, Fontoura et al. (2006) also observed that the most critical stage for leaf area loss is R5, which significantly reduces crop yield with the intensification of the plant's leaf area removal. Silva et al. (2012) found that in all phenological stages productivity is lost as the defoliation degree is intensified.

Knowledge of the habits and biology of insect pests species that occur in each region is essential to avoid crop losses, because during the crop cycle many species of insects can arise, and some of which can increase its population at the extend of being capable to cause losses to farmers, due to reduced production. In this context, Wiest and Barreto (2012), mention that it is essential to have knowledge about the insects that occur in a crop, in order to be programmed to perform phytosanitary treatments and also to predict new pests emergence. Thus, the aim of this study was to report for the first time the *I. dives* coleopterous occurrence in bean crops in the Western Paraná State region.

## MATERIALS AND METHODS

In experiments with the bean crop installed in August 2014, in the cities of Assis Chateaubriand- PR and Palotina-PR, western Paraná state, the presence of a beetle was observed between the months of September and October, occurrence that has not been reported so far in bean crops in this region. The beetle's size was 7 to 9 mm long, with shiny metallic green coloration in its elytra and metallic dark blue on its prothorax. It fell on the ground when touched, as it was dead.

Bean cultivars used in the experiments were IPR Campos Gerais, IPR Tangará and IPR Tuiuiú. When bean plants were among the phenological stages V4 - third trifoliolate leaf, and R5 - flower buds (Didonet and Victoria, 2006; Fancelli and Dourado Neto, 2007) insects present in the experimental area were collected and stored in 70% alcohol, and later were sent for identification in the Invertebrate Zoology section at Natural Sciences Museum from Zoobotanic Foundation of Rio Grande do Sul, Porto Alegre, state of Rio Grande do Sul, Brazil.

## RESULTS AND DISCUSSION

The insect was identified as a coleopterous belonging to the Chrysomelidae family, which species is the *Iphimeis dives* (Germar, 1824). Regarding this species,



**Figure 1.** *Iphimeis dives* coleopterous (Germar, 1824) in bean leaves (*Phaseolus vulgaris*).

Mariconi (1962) mentioned that for a few reasons known it had not been investigated by the entomologists yet, although at that time there were already reports of this insect using various plants as hosts (black wattle, coffee, jabuticaba, orange, velvet bean, kapok, rose, soy, vines). Basso et al. (1974) also reported that this insect attacks the eggplant (*Solanum melongena*). Milléo et al. (2013) mentioned that *I. dives* occur very often in various fruit tree orchards (orange, lemon, tangelo, tangerine, persimmon, apple, nectarine, pear, peach).

Wiest and Barreto (2012), reporting the insect pests evolution on soybean crops in Mato Grosso state, mentioned that in 1988 *I. dives* was considered a secondary pest of this crop. However, in later years there was no record of this pest attacking the soybean crop. These authors further said that since the survey conducted in 2008, this pest was not even framed as a sporadic pest of soybean crops; it was possibly removed from that farming. Though, there are still few information about this insect in the bibliography, and the few quotes found relate to damage caused by the adult ones in some fruit trees. In the experimental field *I. dives* was found feeding on bean leaves (Figure 1), which resulted in decreased plant leaf area (Figure 2). The leaves are responsible for the plant gas exchange and photosynthetic activity, so any factor that interferes with the leaf area can affect productivity (Raven et al., 2007).

According to Acioli et al. (2014), chrysomelids while feeding can cause superficial damage in leaf tissue or even pierce the leaves by making more or less regular and circular shaped holes. When their attack is intense, the leaf is completely perforated, what reduces the photosynthetic capacity, and consequently the production.



**Figure 2.** Damage in bean leaves (*Phaseolus vulgaris* L.) caused by *Iphimeis dives* coleopterous (Germar, 1824).

Oliveira and Ramos (2012) point out that it is important to observe the crop development stage in which the damage occurs. For Acioli et al. (2014) chrysomelids attack can occur at any stage or period of the plant's development, but occurring preferably in younger plants and leaves, which requires greater care in the early stages of plant growth. Quintela and Barbosa (2014) mentioned that when plants are in a higher stage of development, the damage caused by chrysomelids are smaller.

Studies have been performed simulating the attack by defoliator insects in bean crop. For example, Silva et al. (2003), by evaluating defoliation on bean crop, concluded that a defoliation of 25% at 24 days after emergence caused an average decrease of 21.7% in bean yield. Pratissoli et al. (2012) and Schmidt et al. (2010), by evaluating the influence of artificial defoliation to simulate losses in bean production, observed a productivity reduction with increased defoliation levels in virtually all development stages analyzed.

For Pratissoli et al. (2012), this fall of grain yield is due to the reduction in photosynthetically active area, which consequently reduces the amount of photoassimilates produced, affecting negatively the productivity components. Fontoura et al. (2006) also mention that productivity components are adversely affected by the intensity of defoliation.

On the other hand, Quintela and Barbosa (2014) report several studies have indicated that bean can withstand tolerable levels of defoliation (20-66%) without reducing production.

However, variations in responses to defoliation observed in diverse studies demonstrate the existence of genotypic variability, and such results should not be extrapolated to different cultivars (Pratissoli et al.,

2012).

For Morales (2000), it is important to have knowledge of the insect populations of Coleoptera order to develop appropriate management methods, aiming to prevent the population increase of pest species of this group. It is worth noting that the Chrysomelidae family insects are an important group of phytophagous insects that feed on a wide variety of plants. In addition to leaves, adult chrysomelids also consume root, stem, flowers, pollen and anther, thus causing direct damage, or even being able to act as virus transmitters to the plants, causing indirect damage (Acioli et al., 2014) and resulting in decreased productivity.

## Conclusion

This report records the occurrence of *I. dives* coleopterous attacking bean crops (*Phaseolus vulgaris* L.), in the cities of Assis Chateaubriand and Palotina, Western Parana State region. Since it is the first record of this insect in bean crop, it is important to follow up its population in this culture in order to observe the real potential of this species as a pest, as it is cited causing damage to other crops of economic importance.

## Conflict of interests

The author have not declared any conflict of interest.

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