**ISOLATION AND IDENTIFICATION OF *Trichoderma harzianum* Rifai, 1969 ISOLATES, FROM THE VIRGIN FOREST SOIL AND OLD TEA ROOT RHIZOSPHERES AT TEA RESEARCH INSTITUTE, KERICHO COUNTY, KENYA**

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# ABSTRACT

In Kenya, most farmers depend on Tea as a major cash crop for their livelihood. Raising of tea cuttings in the nursery is challenging due to antagonism by various shoot and root pathogens such as *Rhizoctonia* spp*.* and *Pythium* spp. This leads to loss of vigour and production of generally weak seedlings that fail to establish in the final planting field. Some *Trichoderma* species have been known to enhance growth in a wide range of plant species and hence could be used to overcome some of the challenges faced in the raising of tea cuttings. Some strains of *Trichoderma* spp*.* establish robust and long-lasting colonization of the root surfaces penetrating the epidermis of plants. This enhances crop productivity, resistance to abiotic stresses, shoots and root development through enhancement of mineral absorption. The objective of this study was to investigate the effect of *Trichoderma* spp*.* isolates on growth and development of ten selected tea clones developed by Tea Research Institute, Kericho County, Kenya. *Trichoderma* spp. were isolated from forest soil (F) and root rhizospheres of old tea plants (TR) using modified Martin’s Rose Bengal Agar (1950) as selective media. The isolates were cultured, purified, identified, and characterized using morphological, cultural and microscopic characteristics in the Microbiology laboratory. Pure cultures were multiplied using Potato Dextrose Agar (PDA) to obtain sufficient quantities for observations and analysis in the Laboratory. Data were collected in June and August 2019 and recorded. The data were analysed using Microscopic, cultural and morphological characterization of the isolates on PDA media to identify and characterise the isolates. Results showed that *Trichoderma* spp. isolates exist in the virgin forest soil and old tea root rhizospheres.A total of seven fungal isolates were successfully isolated from the soil samples. They were labeled as; T1-2;T2-1;T2-3;T1-1;T3-1;F2-1;F3-1. Five isolates labeled as;T1-2;T2-1;T2-3;T1-1;T3-1; were from old tea rhizospheres .Two isolate labeled as; F2-1 and F3-1 were from the forest soil.It was concluded that after identification of the isolates that all the six fungal isolates were Trichoderma species. They were labelled;T1-2; T2-1;T2-3;T1-1;T3-1;F2-1. One isolate i.e. F3-1 was Pestalotia species. It was, recommended that farmers, agricultural scientists and policy makers could find the *Trichoderma* spp in the virgin forest soil and old tea root rhizospheres for use in raising tea cuttings in the nursery or during research involving *Trichoderma* spp.

KEY WORDS: *Trichoderma* spp -Forest isolate; T4-Standard isolate TR-Tea root Rhizosphere isolate; Clones

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## **Background of the study**

The development of biological control agents of plant pathogens has attracted a significant amount of interest in recent years due to global concerns to conserve the environment and the negative impacts of chemical pollutants on human health worldwide. The research conducted in this field has resulted in the discovery of many potential biocontrol fungal agents of which some have reached the stage of commercialization (Kabaluk, 2010). The ability of some *Trichoderma* spp. to parasitize, kill and destroy plant pathogens have attracted the attention of farmers, agricultural scientists, and policy makers worldwide and hence resulting in accumulation in the recent past of a large body of information on biological control of plant pathogens by some *Trichoderma* spp. (Bissett., 2015).The most recent reports on some of the beneficial microorganisms from different parts of the world have demonstrated their role in the promotion of plant growth and induction of defense response on host plants in addition to antagonistic action on plant pathogens (Contreras, 2016). Inoculation of tea cuttings with different species of arbuscular mycorrhizal fungi (AMF) isolated from different tea rhizospheres in Assam (India) significantly improved the survival of the cuttings (Barthakur, 2005).*Glomus fasciculatum* species was found to be the most efficient and registered the highest shoot length, root length, dry weight and nutrient uptake in tea cuttings. Field experiments conducted in Assam revealed increased leaf harvest in the AMF inoculated plants in comparison to uninoculated ones (Barthakur, 2005).

In Morocco, the Carob tree (*Ceratonia siliqua*) is widely distributed and planted stands in the whole country with an annual production estimated at 26,000 tones. It is an agro-forest-pastoral species having an enormous socio-economic and ecological interest. Research on the effect of double inoculation with endomycorrhizae species and *T.harzianum* on the growth of Carob plants showed that it had a significant effect on the growth of these plants (Zouheir, 2016).

In Kenya investigations carried out on *Trichoderma* spp. have shown their potentials in enhancing the overall growth in tea plants (Wanjiru, 2009). Previous studies carried out at the Tea Research Institute Kericho showed antagonistic properties of some *Trichoderma* species against *Armillaria* fungi in tea establishment (Cheruiyot, 2016). In Kenya there was no research done to find out the effects of *Trichoderma* spp.on any specific tea clone .There was also no nursery-based research that has been done on the screening of commercial tea clones for enhanced growth by *T.harzianum* spp.This study aimed at investigating the nursery based screening of ten selected commercial tea clones (*Camellia sinensis*) for enhanced growth by *T.harzianum* isolates to bridge the identified knowledge gaps.

**Statement of the problem**

Tea is mainly propagated vegetatively using single leaf cutting of about 2.5 cm to 4 cm in length which are then planted in polythene sleeves (Simon, 2014).Tea cuttings take 8-10 months in the nursery. The first 4 months of growth are critical since the plants are under greenhouse conditions and that is the time development of roots and shoots takes place. The next phase which takes another 4 months is the hardening off stage when the plants are exposed to the natural environment for acclimatization (Simon, 2014).It is worth noting that if the plants have not developed roots and shoots during the first phase (4 months) they will not develop in the hardening off stage. The plants will succumb to environmental stress and die regardless of the degree of management (Nyabundi, 2012). In Kenya, during the raising of tea cuttings in the nursery, it has been found that many of the tea cuttings do not develop roots and shoots well. This causes the death of the seedlings when they are transplanted. Since the mycorrhizal organisms have been found to promote good development of roots and shoots in some plants, there was a need to find out the role of *T. harzianum* in promoting growth in tea plants. The present study therefore was initiated with the aim of isolating and identifying *Trichoderma harzianum* Rifai, 1969 isolates, from the virgin forest soil and old tea root rhizospheres at tea research institute, Kericho county, Kenya.

## **Justification and Significance of the study**

Traditional agricultural practices worldwide are increasingly being faced with various problems such as decreased soil fertility due to hazardous chemical pesticides, diseases, pests, droughts, pollution, and global warming (Saba, 2012). When tea cuttings are grown in the nursery under natural conditions, it has been observed that the above-cited factors affect the overall development of roots, shoots, and leaves. Therefore, there was a need for eco-friendly biocontrol agents that could help to resolve some of these problems. Biological control by use of specific micro-organisms that interfere with plant pathogens and pests, is an ecological and nature-friendly approach to overcome the problems caused by chemical methods of plant protection (Harman G. H., 2004).

Microorganisms such as bacteria and fungi are involved in biocontrol activity and the fungi of the genus *Trichoderma* plays a key role in the control of plant disease agents (Saba, 2012). *Trichoderma* strain used as a biocontrol agent against plant pathogenic microorganism act as follows: they colonize the soil and/or parts of the plant, occupying the physical space and prevent the multiplication of the pathogen, they produce cell wall degrading enzymes against the pathogens, they produce plant antibiotics (phytoalexins) that could kill the pathogens, and they promote the plant development by inducing the defensive mechanism of the plant.

This study, therefore, aimed at isolating and identifying *Trichoderma harzianum* Rifai, 1969 isolates, from the virgin forest soil and old tea root rhizospheres at tea research institute, Kericho county, Kenya**.** The findings of this study could be used in overcoming the challenges faced in the raising of tea cuttings in the nursery. It would also be significant to agricultural scientists, farmers, and policy makers in Kenya and worldwide in utilizing *T. harzianum* isolates in tea nurseries as the biocontrol agent of plant pathogens and as biofertilizers. The findings could also benefit agro-based industries in Kenya in developing products based on *T. harzianum* isolates for use against a wide range of plant pathogenic microorganisms.

### **Overall objective**

To isolate and identify *T. harzianum spp* from the virgin forest soil and old tea root rhizospheres.

**Hypothesis of the study**

There are no *T. harzianum spp*. found in the virgin forest soil and the rhizospheres of old tea plants.

**Scope and limitations of the study**

Soil sampling was limited to the elevated areas in a virgin forest and old tea root rhizospheres. The research was carried out for a period of nine months. Laboratory isolation of *Trichoderma* spp.was done in the Soil Microbiology Laboratory at Tea Research Institute. Identification of *Trichoderma* spp. was done using fungal microscopic, cultural and morphological characteristics. The research was also conducted in controlled conditions in the Laboratory.

**Biology and Taxonomy of *Trichoderma* spp.**

The genus *Trichoderma* consists of fungi which are generally soil-dwelling saprophytes. They have been found to be useful in the control of plant pathogenic microorganisms (Sharma, 2014). Members of this genus exhibit the following characteristics; rapid growth rate, produce various antibiotics/phytoalexins (such as gliotoxin and viridin), produce abundant spore, can outcompete other soil microorganisms, and are resistant to agrochemicals (Wanjiru, 2009).This genus belongs to the phylum Ascomycetes, class Sordariomycetes, order Hypocreales, family Hypocreaceae as per (Persoon, 1794). *Trichoderma viride* (Bisby, 1939) was the only species reported by 1968. Later based on morphological characteristics (Rifai, 1969), distinguished nine “aggregate species” as follows:

1. *Trichoderma harzianum* Rifai, 1969
2. *Trichoderma viride* (Bisby, 1939)
3. *Trichoderma hamatum* Bainier,1906
4. *Trichoderma koningii* (Oudem, 1902)
5. *Trichoderma polysporum* Rifai, 1969
6. *Trichoderma piluliferum* Webster & Rifai, 1969
7. *Trichoderma aureoviride* Rifai, 1969
8. *Trichoderma longibrachiatum* Rifai, 1969
9. *Trichoderma pseudokoningii* Rifai, 1969

The nine “aggregate species” are morphologically similar and they cannot be easily separated (Rifai, 1969). The verification of the taxonomy and systematics of the entire genus has been explored by many taxonomists (Kinderman, 1998).Molecular methods such as analysis of the internal transcribed region 1 (ITS1) and the sequencing of the rRNA coding region has been done by Bisset (1991) who recognized four ITS sections:

1. Transcribed section *Hypocreanum* Bisset, 1991
2. Transcribed section *Longibrachiatum* Bisset, 1991
3. Transcribed section P*achybasium* Bisset,1991
4. Transcribed section *Trichoderma* spp.

These sections were distinguished by the differences in conidiophores branching patterns, phialides structure, and shapes of their conidia. One of the main characteristics of transcribed section *Pachybasium* is that phialides are clustered in heads often arising from a broad cell. *Trichoderma* section *Pachybasium* has been further divided into two distinct phylogenetic groups termed as ; phylogenetic group A and phylogenetic group B (Kinderman, 1998);

1. *Trichoderma piluliferum* Webster and Rifai, 1969
2. *Trichoderma polysporum* Rifai, 1969
3. *Trichoderma pubescens* Bisset, 1991

The three above are grouped in the phylogenetic group A while *Trichoderma harzianum* falls into the phylogenetic group B. Molecular methods have substantiated new species concept currently used in the genus. It has also led to the discovery of the new strains (Zimand, 1996). *Trichoderma harzianum species* structurally, bear lateral as well as terminal phialides which are whorled. Their conidia are smooth in texture, rounded in shape and small in size of between 3.6 µm and 4.5 µm in diameter. Their colonies are pale white, thinly cottony that give rise to white tufts which turn green as conidia develop to maturity (Bissett, 1991).

**Soil sample collection and Laboratory culture media preparation**

Fresh soil samples are ideal for isolation of *Trichoderma* spp.. They should be collected randomly from the same location and stored at 40C until they are used (Sagarika, 2017).

The culture media such as Potato Dextrose Agar (PDA) was ideal for fungal growth and was essential for fungal characterization. The basic procedures applied to almost any type of assay or culture requirement for the growth of plant pathogens are mandatory as per the guidelines and protocols of (TRFK, 2017). Generally, the media requirements displayed a wide range of nutritional requirements for fungal growth. They included; water, a source of energy, sources of Carbon, Nitrogen, Sulphur, Phosphorus, some minerals elements (such as Ca2+, Mg2+) some vitamins and growth factors. Microorganisms could also be cultured on solid or liquid media as outlined below as per the guidelines and protocols of (TRFK, 2017).

**Liquid media**: Normally are utilized for physiological studies and assays or growth of large numbers of organisms.

Solid media: They are useful for observations of the characteristic of colonies, isolation of pure cultures and for short-term maintenance or storage of cultures. Usually, the preparation of a solid medium for growth simply includes the addition of 1.5 to 2% agar to a solution of appropriate nutrients.

**Nutrient Agar:** Nutrient Agar is a complex carbohydrate extracted from marine algae that solidifies below temperatures of 450C.Nutrient agar plates are used for isolation and for short term maintenance or storage of cultures (TRFK, 2017).

**Colony-forming unit (cfu):** The term 'colony-forming unit' (cfu); refers to a spore, a clump of spores, a hyphal fragment or one or a clump of vegetative cells if yeast cells are involved. A length of mycelia mass or hyphae can produce various numbers of viable fragments depending on the method used for suspending the sample in combination with the inherent properties of that species and the physiological condition of the fungus (TRFK, 2017).

**Laboratory Sterilization and Aseptic techniques**

**Sterilization:** The term “Sterilization” refers to the process of destroying or physically removing all forms of microbial life which may include vegetative cells, spores, and viruses from surfaces, a medium or an article (Varghese, 2017). The main reasons for sterilization against microorganisms include the following: To prevent transmission of diseases and infections. To prevent contamination by undesirable microorganisms. To prevent deterioration and spoilage of materials by microorganisms

The sterilization methods that could be employed depending on the material which has to be sterilized, the purpose for which sterilization is carried out and the nature of the microorganisms that are to be removed or destroyed. The laboratory safety and sterilization measures provide that all equipment and materials used for culturing and isolation should sterilized as outlined below as per the protocols of (Halgrow, 1999) ;Use of autoclave for steam sterilization at the temperature of 1210C for dilution water and culture broths for at least 15-20 minutes. Oven for dry sterilization at the temperature of 1700 C for pipettes in metal containers for 20-30 minutes.

**Aseptic laboratory techniques:** The laboratory safety and aseptic techniques provides that there should be no cross-contamination of samples and culture media with microorganisms. The general aseptic techniques observed according to the protocol of (Halgrow, 1999) include;

* Washing of hands.
* Cleaning of the benches and working areas with a swab soaked in methylated spirit or 70% ethanol.
* Avoid touching any part of the container, pipette, etc., coming in contact with the sample or cultures.
* Do not remove the lid of a Petri dish or cap of a test tube longer than necessary.
* Lightly flame top of test tubes, ends of pipettes, necks, and stoppers of bottles before and after adding/ withdrawing samples or inocula.
* Transfer culture from one tube to a fresh tube (sub-culturing) using a wire loop sterilized by heating to redness and cooled.
* Always follow all the instructions to prevent contamination and injuries in the laboratory.

**The Laminar air flow chamber**

The Laminar airflow chamber is an apparatus found in the microbiology laboratory. It consists of an air blower in the rear side of the chamber which can produce an air current flowing with uniform velocity along parallel flow lines. It has got an inbuilt special filter system of high-efficiency particulate air filter (HEPA). These filter systems remove particles as small as 0.3 mm from the air entering it (Varghese, 2017). At the front part of the air blower, there lies a mechanism through which air is blown from the blower producing air velocity along parallel flow lines and over the working surface. The principle working mechanism of the laminar airflow is based on the flow of air current of uniform velocity along parallel flow lines which help in the transfer of microbial cultures in aseptic conditions. Air is passed through the system of filters into the enclosure and these filters do not allow any kind of microbe and other particles in the air to enter into the system (Varghese, 2017).

## **Enumeration of colony populations using the spread plate technique**

The spread plate technique can be used in the separation of dilute, mixed population of the microorganisms to obtain the individual colonies that are to be isolated. In this method, a small volume of dilute microbial suspension is transferred to the center of an agar plate and by use of a sterile L-shaped bent glass rod, the mixture is spread evenly over the surface (Varghese, 2017). The Petri dish is spun and at some stage and the single cells will be deposited with the bent glass rod on the agar surface. The agar plate can then be incubated at 37ºC for 24 hours and observed for the dispersed cells which develop into isolated colonies. If the number of colonies become equal to the number of viable organisms in the sample, then the spread plate’s technique can be used to count the microbial population (Varghese, 2017).This technique recommends two methods for use namely:

**For solid media**; it is appropriate to use the plate count method. Enumeration on solid media is done by counting individual colonies that must be well separated. Each colony represents 1 cell and the dilution technique is needed for high concentrations. One millimeter (1 ml) of the sample is added to 9 ml diluents which should be a standard salt solution. Three or more dilution series should be made.

**For liquid media**; the Most Probably Number (MPN) method of estimation is appropriate though it is less accurate than plate count. This method is based on the MPN of units in a sample based on the number of tubes with gas production and volume of inoculums (MPN/100ml) sample. It is a statistical estimate (Halgrow, 1999).

**Isolation and identification of *Trichoderma* spp.**

*Trichoderma* spp. can be isolated from the soil or any other source into PDA plates using the spread plate technique. The suspension is cultured in PDA plates for isolation of the fungi. All the *Trichoderma* spp. growing on PDA plates are sub-cultured repeatedly during isolation into pure cultures (Sagarika, 2017).

During identification procedures of *Trichoderma* spp. isolates, colony morphology, color, the margin on the PDA plate of each isolate is carefully observed and recorded. Microscopic specimens are prepared and using a calibrated phase-contrast microscope, spore shape, size and mycelia width of each isolate are measured (Sagarika, 2017).

**Effects of *Trichoderma* spp. on the growth of tea**

The first experimental use of *Trichoderma* spp. in tea, was initiated in 1983 at Tocklai Tea Research Institute, India with a formulation of Bintab-T being obtained. The pellets of Bintab-T obtained were drilled inside infected tea branch and treated plants showed a marked improvement over untreated controls (Anon, 1983, 1984).Field trials involving the application of *Trichoderma* spp. on planting pits during transplanting of tea seedlings reduced mortality due to root rots (Anon, 1991, 1992). *Trichoderma* spp. application in tea cuttings in the nursery during establishment was found to be enhancing callus development ( healing of wounds) and promote the growth of roots (Anon, 2010, 2011).The cultural practice of pruning being essential agronomic operations carried out in tea plantation exposes the cut surfaces of tea stem making them vulnerable to infections. Copper fungicide is applied to cut surfaces as a protective measures against the entry of *Poria* and other fungi. The tea industry in India currently, uses some *Trichoderma* spp. for the protection of pruning cuts, *fusarium* die back, root diseases, as planting pit mixture and enriching compost (Barthakur, 2005). Native *Trichoderma* spp*.* occurs in tea soils, pruning litter of tea, and also in the air over tea plantation (Debnath, 2016).

In Kenya, it has been reported that *Armillaria* species is a fungus that causes root rot on a wide range both natural and artificial forest hosts leading to high mortality and yield losses of tea plants. It affects many plant species in the highlands and has been a limiting production factor in the tea-growing areas (Cheruiyot, 2016). Yield reductions of up to 50% have been reported on smallholder farms due to *Armillaria* root rot, making it a disease of major economic concern. It has been found that there is a negative interaction and antagonism of *Trichoderma* spp. against *Armillaria* fungus. Some *Trichoderma* spp. isolates perform better than others in reducing *Armillaria* spread and growth in field trials (Cheruiyot, 2016).

**The mechanisms of biological disease control activities by *Trichoderma* spp.**

The frequent use of chemicals such as methyl bromide and fungicide in the control of plant pathogens has caused serious pollution and concerns in environmental conservation. The use of these chemicals is very expensive and their persistent heavy usage is hazardous to the environment (Talukder, 2007). The use of microorganisms as an alternative biological control method of plant pathogens has been considered as a more natural and environmentally acceptable alternative to the chemical control methods (Sagarika, 2017). Therefore, some *Trichoderma* species have been used as an alternative biological control method for phytopathogenic fungi in the control of plant diseases. Some of the strains such as *Trichoderma viridae, Trichoderma polysporum*, *Trichoderma hamatum*, *Trichoderma aureoviridae*, *Trichoderma harzianum* can kill plant pathogens. Some examples which have been observed include the following amongst others (Eziashi, 2006);

*Trichoderma virens* against *Pythium ultimum* infecting cotton and *Rhizoctonia solani* infecting tobacco and *Trichoderma harzianum* against *Verticillium dahliae* infecting potato (TRFK., 2002) *Trichoderma hamatum* for the control of *Pythium* seed rot and *Rhizoctonia* root rot in pea.

The biocontrol of these species is as a result of the action of antagonism through the various mechanisms such as in mycoparasitism (parasitism, hyper parasitism). *Trichoderma* spp. is observed to grow tropically towards hyphae of other pathogenic fungi, coil around them and in the process produce extracellular enzymes that degrade their cell walls. Such enzymes include chitinases, proteases, and glucanases (Haran, 1996). The biological control activities could also be by stimulation of the plants’ defense mechanisms to produce chemical substances that inhibit disease-causing pathogens from multiplication and subsequent infection of the plants. Some investigations have shown that there was an increased level of peroxidases and chitinases in the leaves and roots of *Trichoderma harzianum* treated cucumber plants relative to leaves and roots of not- treated as early as 48 hours after inoculation. That resulted in systematic resistance in the plants as those substances were inhibitory to a wide range of fungi and bacteria (Yedida, 1999). They also stimulate the plant to produce phytoalexins which are antibiotics against a wide range of phytopathogens (Zimand, 1996). When soil is treated with some *Trichoderma* spp.it has been observed that bean leaves became resistant to diseases that are caused by the fungal pathogens, *Botrytis cinerea* and *Colletotrichum lindemuthianum* and also induced systemic resistance in the plants (Bigirimana, 1998).

# MATERIALS AND METHODS

## **The Study area**

The research was conducted at Tea Research Institute (TRI), Kericho station within Timbilil Tea Estate of the latitude of 0° 22’ and 00 28’South; longitude 35° 22’ and 360 12’ East, in Kericho County, Kenya (Fig. 3.1). The area borders Mau forest to the west, about 10km east of Kericho town. It has a mean annual rainfall of 1800 mm and the annual temperate of between 18°C and 230C.It received the solar radiation (MJ m-2 d-1) of 20.72, elevation of 2180 m and the agro-ecological zone is the upper midland (UM). The area has red volcanic soil that is deep, well-drained with  pH ranges of between 5.0 and 6.5 (Kamunya *et al.,* 2012).Kericho County administratively is divided into five sub-counties; Bureti, Soin Sigowet, Kipkelion East,Kipkelion West, and Ainamoi . The main agricultural activity was tea growing which formed the economic mainstay of most households. Other subsistence crops grown include maize, beans sugarcane, bananas, and horticultural crops such as vegetables and fruits. The estate Borders Mau forest which recieves long periods of heavy rainfall between the months of March to June and the months of September to November (Kamunya, 2012).

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STUDY AREA

Figure 3.1 Map of Kenya showing the study area.

Source: Tea Map of Kenya West of the Rift Valley 2016

**SAMPLING AND SAMPLE SIZE**

a) Soil sampling and sample size

An indigenous forest and tea rhizospheres (from nearby tea bushes) were selected. The soil was collected randomly along the transect line at an interval of 10 m. The vegetation was cleared on each identified spot.Using soil auger the soil was collected from a depth of twenty (20) cm. The total number of soi samples collected was recorded. The collected soil was put in Khaki envelopes and labeled as Tea rhizospheres (TR), Forest soil (F). They were transported in a cool field box to the laboratory where they were prepared for use.

**EXPERIMENTAL DESIGN**

**Preparation of soil samples**

In the laboratory, soil samples from each site were mixed thoroughly to obtain a composite sample and from it, a sub-sample was scooped for culturing, isolation and identification of *Trichoderma* spp. Two grams of the composite soil sample from each site (forest and old tea root rhizosphere) was dissolved in 10 ml of distilled water and serial dilutions were made to 10-4  as per guidelines provided by Halgrow, 1999 as illustrated in Fig. 3.2 below:

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Figure 3.2: Serial dilution of soil sample Source: (Halgrow, 1999).

Isolation and purification of the Fungal isolates using the spread plate method

The isolation of *Trichoderma* spp. was done using the Modified Martin’s Rose Bengal-Streptomycin Agar medium (1950). The selective culture media used inhibited the growth of other micro-organisms on culture plates and only allowed *Trichoderma* spp. to grow. The media was prepared and used for culturing *Trichoderma* spp. as shown in Appendix 2. The media was dispensed into the sterilized Petri dishes in the laminar flow to prevent any contamination (Varghese, 2014). Using a bent glass rod, a little amounts (0.1 ml) of the diluted solution of soil samples (10-4) was spread on each plate in three replicates. That was repeated for the other soil dilutions. The plates were incubated at room temperature (25oC) for 5 days. The cultures were observed daily for the germination of fungal spores. Sub-culturing was done repeatedly to purify the cultures to obtain pure cultures for identification. Pure cultures were stored in culture forms in a sterile lamina flow waiting to be used in inoculating tea cuttings in the nursery (Varghese, 2017).

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## **Identification of Forest (F) and Tea Rhizospheres (TR) isolates**

Identification of the *Trichoderma spp.* isolates from the Forest (F) and Tea Rhizospheres (TR) was done using microscopic, cultural and morphological characteristics as follows;

1. The colony appearance at the middle which was concentric or non-concentric.
2. Growth rate based on the number of days to sporulate.
3. The colony/mycelia morphology which was raised or flat and cottony or not cottony.
4. The mycelial structure which was septate or aseptate.
5. The colony and spore pigmentation.

*Trichoderma* spp. (T4) isolates that had been identified and characterized by Tea Research Institute were grown on culture plates and multiplied to obtain sufficient quantities for inoculation as a standard check on the results obtained.

## **The Haemocytometer and Cell Count Technique**

The haemocytometer is a device used to measure the blood cells and also for counting other cells such as the spores and bacteria. The apparatus consisted of a number of chambers. It had a big chamber measuring 1 x 1 x 0.1 mm = 0.1 mm3 in volume with an area of 1 x 1 mm = 1 mm2. The depth of the chamber was 0.1 mm. (1 x 1 x 0.1mm = 0.1 mm3 = 0.0001cm3 = 10-4 cm3 = 10-4 ml). Hence, the spore or bacterial cell counted in the large chamber would be multiplied by 10 4 to give an estimated bacterial cell number/ml (Varghese, 2017). Each of the large chambers had 9 medium-sized chambers measuring 0.2 mm in length, 0.2 mm in width and 0.1 mm in depth with a volume of 0.004 mm3. The medium-sized chamber was divided into 25 small chambers measuring 0.04 mm in length, 0.04 mm in width and 0.1 mm in depth with a volume of 0.00016 mm3 (Varghese, 2017). The spore count (colony forming units) using Haemocytometer was carried out in the laboratory for each isolates forest (F), tea rhizosphere (TR), and standard isolates (T4) in order as shown in appendix 3, to establish the correct concentrations to be applied to each experimental tea cuttings in the nursery (Varghese, 2017).

## **3.10 Laboratory preparation of TR, F, and T4 suspension for inoculation**

After spore count using haemocytometer, an estimate of the number of spore (colony forming units) for each isolate was arrived at. The solution were made accordingly to inoculate all the tea clones in the nursery as shown in Appendix 4. Each tea clone was planted in polythene sleeves, inoculated and left to grow in a specially prepared seedling bed.

## **DATA COLLECTION AND ANALYSIS**

Identification and characterization of *Trichoderma* spp. isolates were done through laboratory observation and analysis of fungal isolate growth characteristics such as colony appearance at middle, colony morphology, mycelia structure, colony pigmentation ,colony margin appearance, and growth rate in days as per the protocol of (Siew, 2013).

## **RESULTS**

### **Isolation of Trichoderma spp.**

A total of seven fungal isolates were successfully isolated from the soil samples, labeled as T1-2; T2-1; T2-3; T1-1; T3-1; F2-1; and F3-1. Five fungal isolates labeled as T1-2, T2-1, T2-3, T1-1, and T3-1 were from old tea root rhizospheres while two isolate labeled as F2-1 and F3-1 were from the forest soil. The isolates were as shown in Plate 4.1 below.

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Plate 4.1 Photo plates of pure cultures of TR isolates from Tea rhizospheres. A-C are isolates from old tea root rhizospheres; D-E, are isolates from the forest soil. Arrows indicate the fungal cultures in culture plates.

### **Pure cultures of the forest isolates (F) from the forest soil**

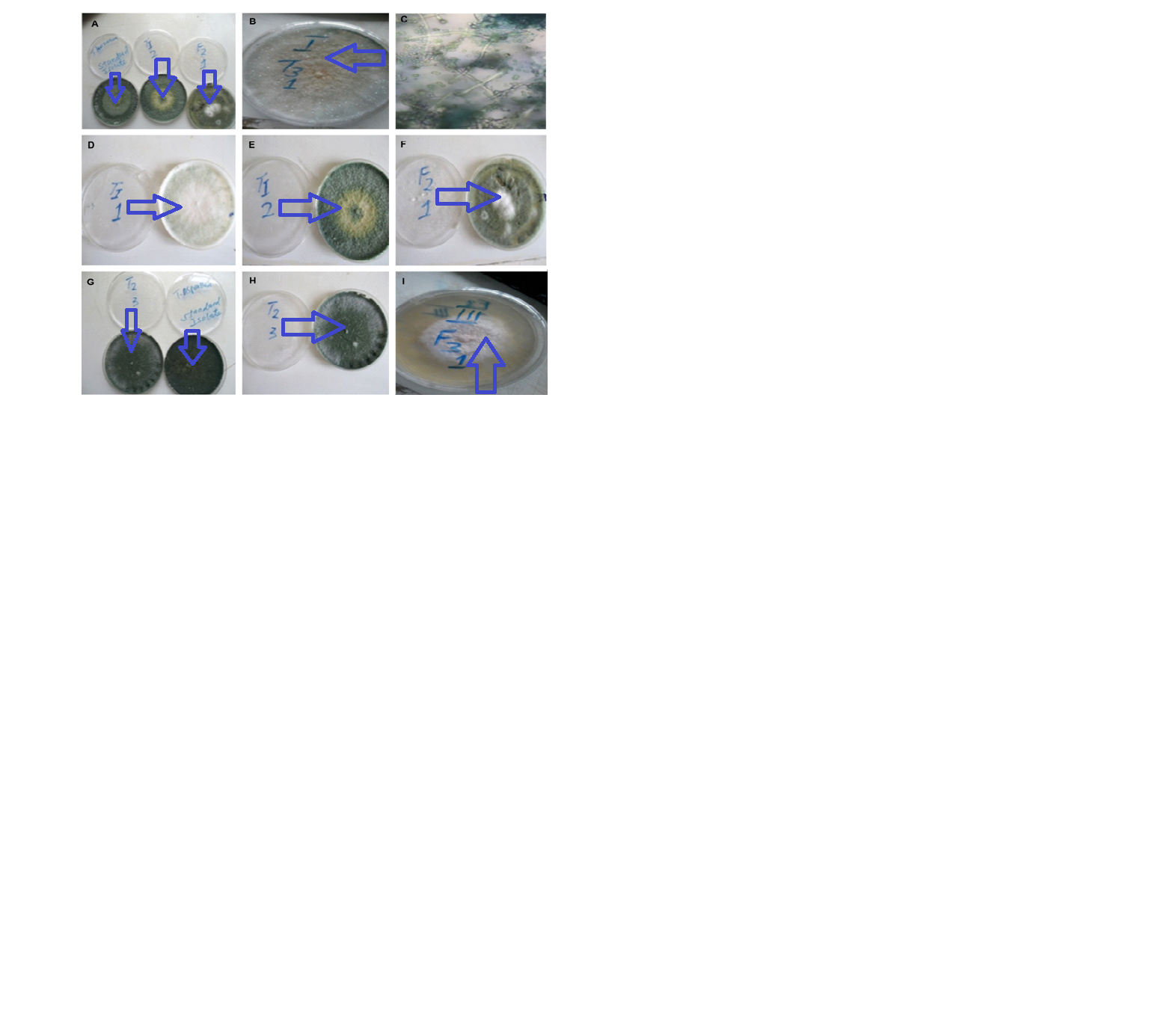
Laboratory identification carried out using microscopic, cultural and morphological characteristics showed that the six fungal isolates labeled as; T1-2; T2-1; T2-3; T1-1; T3-1; and F2-1 were *Trichoderma* species, while one isolate named as F3-1; was *Pestalotia* species ( see Plate 4.2).****

Plate 4.2 Photo plates of pure cultures of F isolates from the forest soil. A-F, *T. harzianum* isolates from forest soil (C-shows branching pattern of *Trichoderma* spp. conidiophores); isolate of *T. asperellum* G-H; Isolate of *Pestalotia* spp. I,, Arrows indicate fungal cultures in culture plates

### 

### **Cultural characterization of the isolates**

Based on the growth characteristics, cultural and microscopic features of the isolates, the fungal isolates were identified as *Trichoderma harzianum* (T1-2; T2-1; T1-1; T3-1; F2-1); *Trichoderma* *asperellum* (T2-3); and *Pestalotia spp. (*F3-1) as shown in Table 4.1.

Microscopic,cultural and morphological characterization of the isolates on PDA media.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Isolates | | Colony appearance | Colony  morphology | Mycelia structure | Colony pigmentation | Colony  margin appearance | Growth rate in days |
| 1 | T1-2 | concentric | -Erect; cottony | Aseptate | -Creamy underside;white colony | Smooth | 4 |
| 2 | T2-1 | concentric | Erect;cottony | Aseptate | Creamy underside; white colony | Serrated | 4 |
| 3 | T2-3 | Non-concentric | Erect; cottony | Aseptate | Creamy underside; Green colony | Serrated | 4 |
| 4 | T1-1 | concentric | Erect;cottony | Aseptate | Creamy underside; Green colony | Serrated | 4 |
| 5 | T3-1 | Non-concentric | Erect;cottony | Aseptate | Creamy underside; Green colony | Serrated | 4 |
| 6 | F2-1 | concentric | Erect;Not cottony | Aseptate | Creamy underside; Green colony | Serrated | 4 |
| 7 | F3-1 | concentric | -Erect;Not cottony | Septate | Creamy underside;Orange colony | Serrated | Over 5 |

### **Cultural growth rates of the isolates on PDA medium.**

Based on the growth rate of the isolates, *Trichoderma* spp. were identified based on their characteristic fast growth and ability to colonize the 9 mm Petri dish in five days.It was found out that when the isolates were cultured on PDA media at room temperature (25°C), nearly all the isolates had the same growth rate except isolates F3-1.Isolates T2-3, F2-1, T3-1, T1-1, T2-1, and T1-2 grew faster than the rest of the other isolates at 25°C on PDA media. Their fast growth rates were mostly noted after 2 days (48 hours) which was a characteristic feature of *Trichoderma* spp. However, F3-1 isolates grew slightly slower than any other isolates at 25°C on PDA and did not fully colonize the 9 mm PDA plate after four days. All the isolates except F3-1 fully colonized the PDA plates on the fourth day. In that case, F3-1 could be clearly distinguished from the other isolates since it showed the slowest growth rate.

The Average Growth rate of the forest soil and Tea rhizosphere isolates on PDA media (mm/day).

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ISOLATES | DAY 1 | DAY 2 | | | DAY 3 | DAY 4 | | DAY 5 | | DAY 6 | |
| REPS |  | |  |  | | |  | |  | |  | |
| T2-3 | 4 | | 26 | 62 | | | 82 | | 82 | | 82 | |
| F2-1 | 4 | | 25 | 64 | | | 82 | | 82 | | 82 | |
| T3-1 | 4 | | 30 | 65 | | | 82 | | 82 | | 82 | |
| T1-1 | 4 | | 28 | 64 | | | 82 | | 82 | | 82 | |
| T2-1 | 4 | | 28 | 60 | | | 82 | | 82 | | 82 | |
| T1-2 | 4 | | 30 | 64 | | | 82 | | 82 | | 82 | |
| F3-1 | 4 | | 7 | 16 | | | 22 | | 35 | | 49 | |

### **Spore count (cfu) using Haemocytometer**

The spore count (colony forming units) using Haemocytometer was carried out in the laboratory for each isolates forest (F), tea rhizosphere (TR), and standard isolate (T4).

**Spore count in TR isolates from old tea rhizospheres.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| S/no. |  | Trial I | Trial II | Average |  |
| 1 |  | 12 | 11 | 11.5 |  |
| 2 |  | 11 | 12 | 11.5 |  |
| 3 |  | 14 | 12 | 13 |  |
| 4 |  | 10 | 12 | 11 |  |
| 5 |  | 9 | 10 | 9.5 |  |
| Total |  | 56 | 57 | 56.5 |  |

From the table, (56/5); 11.3 spores (colony forming units) were contained in 0.004 ml of the Haemocytometer

Therefore; in 10 ml of the diluted solution, there were {10 ml/0.004 ml x 11.3 spores}

=28,250 spores in 10 ml of the stock solution diluted to 10-2

The concentration of 10 ml of the stock solution was;

= (28,250 spores x 100) = 2,825,000 Spores

Therefore; the concentration of 150 ml of the stock solution made contained;

= (2,825,000 spores x 150) spores (cfu)

= 423,750,000 spores (cfu)

The concentration of the stock solution per ml was = (423,750,000/150)

= 2.825 x 106cfu/ml

Spore count in F isolates from the forest soil.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| S/no. | Trial I | Trial II | Average |  |
| 1 | 13 | 8 | 10.5 |  |
| 2 | 14 | 11 | 12.5 |  |
| 3 | 12 | 8 | 10 |  |
| 4 | 11 | 8 | 9.5 |  |
| 5 | 12 | 8 | 10 |  |
| Total | 62 | 43 | 52.5 |  |

From the table, (52.5/5) 10.5 spores (colony forming units) were contained in 0.004 ml of the Haemocytometer.

Therefore in 10 ml of the diluted solution, there were {10 ml/0.004 ml x 10.5 spores}

=26,250 spores in 10 ml of the stock solution that was diluted to 10-2

The concentration of 10 ml of the stock solution was;

= (26,250 spores x 100)

= 2,625,000 spores

Therefore; the concentration of 150 ml of the stock solution made contained;

= ( 2,625,000 spores x 150) spores (cfu)

= 393,750,000 spores (cfu)

The concentration of the stock solution per ml was;

= ( 393,750,000 /150)

= 2.625 x 106 cfu/ml

Spore count in T4 isolates; Standard isolates.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| S/no. | Trial I | Trial II | Average |  |
| 1 | 33 | 17 | 25 |  |
| 2 | 25 | 15 | 20 |  |
| 3 | 15 | 17 | 16 |  |
| 4 | 32 | 18 | 25 |  |
| 5 | 23 | 19 | 21 |  |
| Total | 128 | 86 | 107 |  |

From the table, (107/5) 21.4 spores (colony forming units) were contained in 0.004ml of the Haemocytometer

Therefore, in 10ml of the diluted solution, there were {10 ml/0.004 ml x 21.4 spores}

=53,500 spores in 10 ml of the stock solution that was diluted to 10-2

The concentration of 10 ml of the stock solution was;

= ( 53,500 spores x 100)

= 5,350,000 spores

Therefore; the concentration of 150 ml of the stock solution made contained;

= ( 5,350,000 spores x 150) spores (cfu)

= 393,750,000 spores (cfu)

The concentration of the stock solution per ml was;

= ( 802,500,000/150)

= 5.35 x 106cfu/ml

**DISCUSSIONS**

**Sampling Technique and sample size determination**

An ideal soil sample for isolation of Trichoderma was freshly collected from the same location and stored at 40C in the laboratory until used. That was to prevent possible denaturing of the isolates due to extreme ambient temperature conditions (Sagarika,2017).

**Isolation of Trichoderma spp.**

A total of seven fungal isolates were successfully isolated from soil samples, namely T1-2; T2-1; T2-3; T1-1; T3-1; F2-1; F3-1.Five fungal isolates were from old tea rhizospheres. They were; T1-2; T2-1; T2-3; T1-1; T3-1 while two fungal isolates isolate F2-1 and F3-1 were from the forest soil. Laboratory identification carried out using microscopic, cultural and morphological characteristics showed that all the six fungal isolates T1-2; T2-1; T2-3; T1-1; T3-1; F2-1 were *Trichoderma* species (Plate 4.1; A-F).While isolate T2-3 had non-concentric mycelia growth hence; identified as *T. asperellum* species (Plate 4.2; G-H). While one isolate; F3-1 was *Pestalotia* species (Plate 4.3; I). Those were the only fungal isolates obtained. However, other isolates such as *Trichoderma viride*, *Trichoderma hamatum, Trichoderma koningii, Trichoderma polysporum* Rifai, (Wanjiru, 2009)

**Growth rates of the isolates on PDA Medium**

The results obtained showed that when the isolates were cultured on PDA media at room temperature (25°C), the six isolates T2-3; F2-1; T3-1; T1-1; T2-1; T1-2 had similar growth rate except for isolates F3-1. Their fast growth rates were mostly noted after two days which was a characteristic feature of *Trichoderma* spp. (Sagarika, 2017).The growth rate was therefore used to identify the isolates as *T.harzianum* species*.* However; F3-1 isolates grew slowly than any other isolates at 25°C on PDA media and did not fully colonize the 9 mm PDA plate after four days. All the isolates except F3-1 fully colonized the PDA plates on the fourth day and could be distinguished from the other isolates as *Pestalotia* species due to its slow growth rate see Plate 4.4; I and table 4.5.

**Identification of Trichoderma spp. isolates**

Morphological characterization remains as a potential method to identify *Trichoderma* species (Samuels, 2002). Therefore, morphological, cultural and microscopic features of *Trichoderma* spp. isolates were used in the identification of the species. The growth rate of *Trichoderma* spp. isolates on PDA at 25°C was studied and established that they had almost similar growth rate at that temperature (25°C). When the cultures were compared with the standard isolates for identifications, it was observed that they were morphologically similar in term of color, growth rates and the general characteristics as described in (Plate. 4.1, 4.2)

**Colony pigmentation**

The microscopic features of *Trichoderma* spp. isolates were observed under a light microscope. The colours of all *Trichoderma* spp.were found to be green which was a characteristic feature of *Trichoderma spp.* The different intensities of green colours ranging from light green, yellowish-green, dark green to the greyish-green of mature conidia observed on plates 4.1 and plate 4.2 and under the light microscope were all the characteristic features of *Trichoderma* spp*.* as described by Sagarika, (2017).

**Branching pattern of conidiophores**

The branching patterns of Conidiophores of six isolate T1-2, T2-1, T2-3, T1-1, T3-1, and F2-1 had paired primary branches (Plate 4.1, 4.2) which were similar. Isolates F3-1 (*Pestalotia* spp.) had lateral side branches produced from the main branches which were not paired, a characteristic branching pattern of *Pestalotia* spp. (Figs. 4.1, 4.2). These fitted the descriptions of the species by Sagarika, (2017).

**Colony morphology**

Colony characters of the isolates were studied using 5 days old PDA cultures which were incubated at room temperatures (25°C).It was observed that all the isolates grew well and formed conidia within five (5) days. The conidia production in plate 3 (T1-2) formed concentric rings and the color of mature conidia was yellowish-green. Colony appearance was erect and cottony with aseptate mycelia. The isolate was identified as *T.harzianum* when compared with the standard isolate T4 (see Plate. 4.1, 4.2). In Plate 4.1A(T2-1), it had mature conidia which were greyish green, and concentric rings with a serrated margin. It also had aseptate mycelia and therefore was identified as *T.harzianum.* The conidia in Plate 4.1A(T2-3) was non-concentric at the centre of the colony dark green in color with aseptate mycelia. When compared with the standard isolate, it was identified as *T. asperellum* as per the protocols of; Siew,(2013). The isolates in Plate 4.1, B **(**T1-1) had greyish-green conidia which formed pustules. They were also arranged in concentric rings which were erect and cottony. The mycelia were aseptate and hence identified as *T.harzianum*.In the colonies of Plate. 4.1, C(T3-1), no concentric rings were observed. Their conidia productions were restricted to the centre of the colonies, diffused, and appeared yellowish green. Mycelia were aseptate and were identified as *T.harzianum.* In Plate. 4.1, D(F3-1) conidia were orange to pink in color, formed concentric rings which were erect cottony with septate mycelia and grew very slowly. It was identified as *Pestalotia* spp. (Plate. 4.1, 4.2). The isolates in Plate. 4.1, E (F2-1) were light green and formed pustules, arranged in concentric rings which were erect and cottony. Mycelia were aseptate and were identified as *T.harzianum*.

## **CONCLUSION AND RECOMMENDATIONS**

The research findings showed that there were *T.harzianum* found in forest soil and rhizospheres of old tea bushes. That was confirmed when a total of seven *Trichoderma* spp. isolates were isolated. Further research to be carried out on isolation of other *Trichoderma spp*. not covered by the research. Molecular methods be used to characterize the isolates as the convention methods may not give the correct identity of the isolates.

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EFFECT OF *TRICHODERMA* ISOLATES ON ROOT GROWTH OF DIFFERENT TEA (*CAMELLIA SINENSIS)* CLONES; NURSERY BASED STUDY

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**ABSTRACT**

Most farmers depend on tea as a cash crop and raising it is challenging due to pathogens and other factors which lower the vigour hence production of weak plantlets. Some *Trichoderma species* promote growth in a wide range of plant species by establishing robust and long-lasting colonization of the root surfaces penetrating into the epidermis and promote roots development; crop productivity and resistance to abiotic stresses. The objectives of this study were to investigate the effect of *Trichoderma spp*. isolates on development of roots and root biomass of ten selected clones. The *Trichoderma* spp. was isolated from forest soil (F) and tea root rhizospheres (TR) using modified Martin’s Rose Bengal Agar. Standard isolate, T4 was from stock cultures. They were cultured, purified and characterized using cultural morphological, and microscopic characteristic. Pure cultures were multiplied using Potato Dextrose and 1ml (2.0 x 106 cfu/ml) suspension used for inoculation. Experiments were set up with controls using DAP fertilizers in a randomized complete block design with three replicates. Observations were made after 120 days where data was collected in June and August 2019. Data was analysed using Statistical Analysis Software (2018) and Two-way analysis of variance (ANOVA). Results showed the isolates significantly (P≤0.05) enhanced development of root and root biomass in the treated tea clones. It was concluded that *Trichoderma spp*. Promote growth in different tea clones and hence, is recommended for use when raising cuttings in the nursery.

KEY WORDS: *Trichoderma* spp -Forest isolate; T4-Standard isolate TR-Tea root Rhizosphere isolate; Clones

**Introduction and Background to the Study**

The development of biological control agents of plant pathogens has attracted significant amount of interest in the recent years due to global concerns to conserve the environment and the negative impacts of chemical pollutants on human health worldwide. The research conducted in this field has led to the discovery of many potential fungal biocontrol agents some of which have reached the stage of commercialization [5].The ability of some *Trichoderma spp*. to parasitize and kill destructive plant pathogens have attracted attention of agricultural scientists, farmers and policy makers worldwide and hence; a large body of information on biological control of plant pathogens have accumulated in the recent past [2].

The most recent reports on some of the beneficial microorganisms from different parts of the world have demonstrated their role in the promotion of plant growth and induction of defenses response on host plants in addition to antagonistic action on plant pathogens [4]. Inoculation of tea cuttings with different species of Arbuscular Mycorrhizal Fungi isolated from different tea rhizospheres in Assam (India) significantly improved the survival of the cuttings [1]. *Glomus fasciculatum species* was found to be the most efficient and had the highest shoot length, root length, dry weight and nutrient uptake in tea cuttings. Field experiments conducted in Assam revealed increased leaf harvest in the Arbuscular Mycorrhizal Fungi inoculated plants in comparison to the control [1]. In Morocco, the Carob tree (*Ceratonia siliqua)* is an agro-forest-pastoral species having an enormous socio-economic and ecological interest. Research on the effect of double inoculation with endomycorrhizae species and *Trichoderma harzianum species* on the growth of Carob plants showed a significant effect on the growth of these plants [11].

In Kenya investigations carried out on *Trichoderma spp.* have shown their potentials in enhancing the overall growth in tea plants [10]. Research carried out at Tea Research Institute showed antagonistic properties of some *Trichoderma* species against *Armillaria spp.* fungi in tea establishment [8], [9] & [ 3]. However, no research has been done on the effects of *Trichoderma* spp. on any specific tea clone. This research was aimed at carrying out an investigation on the nursery-based screening of ten selected commercial tea clones inoculated with *Trichoderma spp.* isolates for enhanced growth of roots; the number of roots and biomass to bridge the identified gaps.

# MATERIALS AND METHODS

## Experimental design

The research was carried out at Tea Research Institute nursery section for a duration of 120 days. The experiments were set up in a Completely Randomized Block Design (CRBD) in which tea clones were planted in polythene sleeve and randomly placed in the experimental blocks in the nursery. The experiments involved 7 treatments namely; Tea Rhizosphere (TR) +DAP; Forest (F) + DAP; Standard (T 4) +DAP; Tea Rhizosphere (TR only); Standard (T4 only) and control (DAP only) in three replicates.

**Source of *Trichoderma spp.* isolates**

The standard *Trichoderma harzianum* isolates T4; was obtained from the stock cultures in the TRI laboratory through sub culturing in Potato Dextrose Agar (PDA) while isolates; F and TR were obtained from virgin forest soil and old tea root rhizospheres respectively, through isolating using Martin’s Rose Bengal Agar (1950). Identification and characterization were done in the laboratory using microscopic, cultural and morphological characteristics. The isolates were multiplied on PDA and appropriate concentrations were obtained for inoculating all the experimental tea clones in the nursery.

**Source of Tea Clones**

Tea clones used for the experiments were TRFK 6/8; TRFK 7/9; TRFK 31/8; TRFK 56/1; TRFK 301/4; TRFK 303/577; TRFK 7/3; TRFK 306/1; TRFK 597/1; TRFK 704/2, their cuttings obtained from the Tea Research Institute-Timbilil Estate mother bushes. Each cutting had two buds and a leaf obtained from mother bush 2.5 cm to 4 cm in length. The Criteria used to select ten clones for the study involved sampling of 10 clones out of 59 commercial clones developed by Tea Research Institute. The 10 samples represented the popular commercial clones which were the standard check for adaptability, high quality and high yielding. Selection of the ten Clones was based on; Yield potential kg/mt/ha/yr.; Quality index of black tea; Rooting of cuttings; Current utilization status [6].

***Trichoderma harzianum species isolates; T4; TR; and F spore harvesting***

The method adopted for harvesting of *Trichoderma species* spores from mature cultures was by scrubbing off using a sterile glass slide. The harvested spores were counted using haemocytometer and the concentration adjusted to 2.0 X 106 colony forming units per ml with sterile distilled water.

***Nursery Bed Establishment***

Polythene sleeves (10 cm diameter by 26 cm long) were used to pot nursey soil comprising of a mixture of both topsoil and subsoil mixed with DAP fertilizers with the application rate recommended and adopted by the Tea Research Institute (600g of DAP per 1,000,000 cm3 volume of soil). The sleeves were filled with the nursery soil, cuttings were planted and watered and covered with polythene sheet. There were 10 different clones per plot each with Replicate 1(R1); Replicate 2 (R2) and Replicate 3 (R3) respectively. Each replicate was further replicated three time to give a total of ninety (90) plantlets per plot. There were seven (7) plots each with ninety plantlets to give a total of 630 plantlets in the experimental plots in the nursery. Tinder net was used to provide 60% shading in order to reduce the impact of direct sun.

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***Inoculation with Trichoderma spp. isolates***

The *Trichoderma* treatments**, T. *harzianum***T4; TR; and F were applied onto each sleeve using a pipette to enhance colonisation by the inoculum. Two millilitres of spore suspension containing 2.0 X 106 cfu was introduced into each respective sleeve. A control experiment was set up using (DAP only) for each clone.

***Data Collection;***

Growth measurements on root lengths by destructive sampling and getting the length using a ruler &, number of roots and Biomass were taken randomly after 120 days for each tea clones. Biomass (dry weights) were recorded after drying the plant materials and weighing until the weight became consistently constant. Details of the data collected were recorded on a designed table for analysis and drawing of general conclusion.

***Data Analysis***

The collected data was subjected to Statistical Analysis Software (SAS version 9.0) for analysis and drawing of general conclusions based on the results obtained.

# RESULTS AND DISCUSIONS

The interaction of Treatment (T) and Clones (C) in table 1 showed varied results when; 10 different clones of tea were subjected to 7 different treatments, to test on their length of roots after 60 days.

**Effects of the treatments on root lengths;** From the findings the root length development did vary significantly (LSD P≤0.05) in all the treatments. Treatments F ONLY, TR ONLY and T4 ONLY enhanced the mean root length development (2.3a) compared to their controls (DAP only). A similar effect of the treatments was also observed after 120 days of growth.

**Interaction of treatments with the clones on root length development;**

Growth and survival of clone TRFK 6/8, TRFK 7/9, TRFK 56/89, TRFK 304/1/, TRFK 597/1, and TRFK 704/2 were enhanced at their initial stage of establishment (Table 1). After 120 days of growth, the findings showed that the treatments enhanced growth in clones TRFK 7/9, TRFK 56/89, TRFK 306/1, TRFK 597/1, and TRFK 704/2 (Table 2).

From the findings of (Barthakur, 2005) [1] inoculation of tea cuttings with different species of arbuscular mycorrhizal fungi (AMF) isolated from different tea rhizospheres in Assam (India) significantly improved the survival of the cuttings. *Glomus fasciculatum* species was found to be the most efficient and registered the highest shoot length, root length, dry weight and nutrient uptake in tea cuttings. Growth and survival of the cuttings from the above finding showed a common trend.

Research conducted by (Zouheir, 2016) [11],showed that double inoculation with endomycorrhizae species and *T.harzianum* on the growth of Carob plants had a significant effect on the growth of these plants. These finding (Table 1 & 2) also showed a common trend with those of other researchers.

**Effects of the treatments on the number of roots;**

The number of roots did vary significantly (P≤0.05) in all the treatments when 10 different clones were subjected to 7 different treatments, to test on their number of roots after 60 days (Table 3). Treatments; TR only; T4 only; and F only enhanced growth root and root biomass and thus recorded the highest number of roots compared to the control treatments. Treatments; F+DAP; TR+DAP; T4+DAP; and Control also did not exhibit significant variation in their means and showed low number of roots. The same trend was also observed after 120 days (Table 4)

**Interaction of treatments with the clones on the number of roots;**

The interaction of Clones with Trichoderma spp showed varied results with clone TRFK 56/89, TRFK 306/1 and TRFK 704/2 producing the highest number of roots after 60 days (Table 4). The same trend was observed after 120 days when clone TRFK 704/2 produced the highest number of roots, followed by clones TRFK 56/89 and TRFK 7/9 respectively. Clones TRFK 303/89; TRFK 301/4; TRFK 597/1; TRFK 6/8 and 306/1 showed moderate numbers of roots. Clone TRFK 7/3 had the lowest root numbers (Table 4).

Wanjiru (2009) pointed out in his studies [10] that Trichoderma spp inoculant improve root development in tea cuttings by 50%. Therefore, the results and finding of the study compared with those of other researchers showed a common trend.

**Effects of the treatments on the dry weights;**

The findings when 10 different clones were subjected to 7 different treatments to test on their dry weight also showed a common trend after 60 days with treatments TR ONLY, F+DAP and TR+DAP, T4+DAP and F only having the highest mean dry weight (Table 5).A similar trend was observed after 120 days ( Table 6) where treatments; TR+DAP; and F+DAP, had the highest dry weight followed by F ONLY; and T4 only respectively. Treatments; Control (DAP ONLY) showed the lowest dry weight.

**Interaction of treatments with the clones on their dry weights;**

The interaction of Clones with Trichoderma spp showed varied results with clone TRFK 306/1 and TRFK 301/4 producing the highest mean dry weight followed by clones TRFK 31/8; TRFK 597/1; and TRFK 6/8 respectively. Clones TRFK 56/89; TRFK 704/2 and TRFK 7/9 showed moderate mean dry weight. Clone TRFK 7/3 recorded lowest mean dry weight (Table 5). After 120 days of growth it was observed that clones TRFK 301/4 and TRFK 704/1 had the highest mean dry weight followed by Clones TRFK 38/1, TRFK 56/89, TRFK 306/1 and TRFK 597/1respectively with Clone TRFK 7/3 recording the lowest mean dry weight.

Investigations done by Wanjiru (2009) showed that Trichoderma spp inoculant improved shoot and root development and root dry weight by 83.78% and 50% respectively [10]. He further argued that Trichoderma spp isolates +DAP fertilizers interactions with tea improved shoot and root dry weights. The results of the investigation showed a common trend as Wanjiru (2009) and other researchers pointed out.

Table 1 The effects of *T. harzianum* on the growth of root lengths after 60 days.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| CLONES | TRFK  6/8 | TRFK  7/9 | TRFK 31/8 | TRFK  56/89 | TRFK  301/4 | TRFK  303/577 | TRFK 7/3 | TRFK 306/1 | TRFK  597/1 | TRFK  704/2 | mean treatments |
| Treatments F+DAP | 3.9  (1.7) | 3.6  (1.73) | 6.5  (2.1) | 1.1  (1.13) | 4.1  (1.81) | 0.0  (0.69) | 0.9  (1.1) | 0.5  (0.9) | 1.7  (1.3) | 1.8  (1.33) | 1.4  b |
| TR+DAP | 1.8  (1.3) | 3.4  (1.68) | 1.2  (1.1) | 2.1  (1.41) | 0.0  (0.69) | 0.0  (0.69) | 0.0 (0.7) | 0.3  (0.8) | 1.5  (1.24) | 6.2  (2.1) | 1.2  b |
| T4+DAP | 0  (0.69) | 0.9  (1.06) | 0.0  (0.6) | 2.8  (1.56) | 4.0  (1.79) | 0.0  (0.69) | 1.5  (1.3) | 2.7  (1.5) | 1.6  (1.29) | 6.8  (2.17) | 1.3  b |
| Control(DAP) | 2.5  (1.5) | 0.6  (0.96) | 1.4  (1.2) | 3.0  (1.6) | 3.0  (1.6) | 1.1  (1.13) | 0.2  (0.8) | 1.0  (1.1) | 2.3  (1.46) | 1.3  (1.2) | 1.2  b |
| F ONLY | 4  (1.8) | 9.0  (2.4) | 7.4  (2.2) | 10.6  (2.5) | 10.6  (2.5) | 8.6  (2.36) | 2.9  (1.6) | 8.5  (2.3) | 8.8  (2.38) | 11.9  (2.6) | 2.3  a |
| TR ONLY | 10.6  (2.5) | 10.1  (2.4) | 6.2  (2.1) | 12.7  (2.6) | 6.2  (2.1) | 8.5  (2.35) | 1.5  (1.3) | 8.6  (2.3) | 11.2  (2.5) | 9.7  (2.46) | 2.3  a |
| T4 ONLY | 8.1  (2.3) | 11.3  (2.5) | 6.2  (2.1) | 8.4  (2.34) | 10.3  (2.5) | 10.3  (2.51) | 1.9  (1.4) | 8.0  (2.3) | 8.8  (2.38) | 8.7  (2.37) | 2.3  a |
| Mean clone | 1.7ab | 1.8ab | 1.7b | 1.9ab | 1.9ab | 1.6b | 1.1c | 1.6b | 1.8ab | 2.0a |  |
| C.V (%) | 35.02 |  |  |  |  |  |  |  |  |  |  |
| LSD (P≤0.05) | |  |  |  |  |  |  |  |  |  |  |
| Treatments | 0.30 |  |  |  |  |  |  |  |  |  |  |
| clones | 0.37 |  |  |  |  |  |  |  |  |  |  |

**Table 2: Effects of treatments on growth of root lengths after 120 days**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Tea clones and root length measurement (cm) | | | | | | | | | |  | |
| **Treatments** | TRFK6/8 | TRFK7/9 | TRFK  31/8 | TRFK 56/89 | TRFK 301/4 | TRFK 303/577 | TRFK  7/3 | TRFK306/1 | TRFK  597/1 | TRFK704/2 | mean treats | |
| Treatment  F+DAP | 2  (1.47) | 17  (2.9) | 8  (2.2) | 7  (2.24) | 2  (1.47) | 1  (1.12) | 1  (1.12) | 3  (1.53) | 8  (2.2) | 15  (2.83) | 1.93c | |
| TR+DAP | 5  (1.9) | 13  (2.7) | 2  (1.47) | 16  (2.87) | 5  (1.9) | 14  (2.7) | 0  (0.7) | 7  (2.21) | 3  (1.53) | 12  (2.6) | 2.08c | |
| T4+DAP | 14  (2.8) | 7  (2.1) | 6  (2.1) | 11  (2.56) | 19  (3.04) | 6  (2.05) | 6  (2.0) | 17  (2.9) | 3  (1.53) | 13  (2.69) | 2.51b | |
| F ONLY | 15  (2.8) | 14  (2.7) | 14  (2.7) | 16  (2.87) | 20  (3.09) | 16  (2.87) | 14  (2.7) | 16 (2.87) | 16 (2.87) | 12 (2.6) | 2.83ab | |
| TR ONLY | 16  (2.9) | 17  (2.9) | 17  (2.9) | 17 (2.92) | 16 (2.87) | 13  (2.74) | 11  (2.6) | 16  (2.87) | 16 (2.87) | 17 (2.9) | 2.88a | |
| T4 ONLY | 12 (2.6) | 16 (2.87) | 14 (2.7) | 17 (2.9) | 18 (2.98) | 17  (2.9) | 3  (1.53) | 16 (2.87) | 16 (2.87) | 15 (2.83) | 2.72ab | |
| **CONTROL (DAP only)** | 11  (2.5) | 12  (2.6) | 2  (1.47) | 14  (2.7) | 1  (1.12) | 2  (1.47) | 4  (1.7) | 2  (1.47) | 2  (1.47) | 6  (2.14) | 1.90c | |
| Mean clone | 2.46bc | 2.71ab | 2.22c | 2.74a | 2.35bc | 2.26c | 1.81d | 2.45abc | 2.40abc | 2.68ab |  | |
| C.V (%) | 26.28 |  |  |  |  |  |  |  |  |  |  | |
| LSD (P≤0.05) |  |  |  |  |  |  |  |  |  |  |
| Treatment | 0.32 |  |  |  |  |  |  |  |  |  |  |
| clones | 0.39 |  |  |  |  |  |  |  |  |  |  |

Table 3 The effects of *T. harzianum* on growth of the number of roots after 60 days.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| CLONES | TRFK  6/8 | TRFK  7/9 | TRFK  31/8 | TRFK  56/89 | TRFK  301/4 | TRFK  303/577 | TRFK  7/3 | TRFK  306/1 | TRFK  597/1 | TRFK 704/1 | mean treatments |
| Treatments  F+DAP | 2.8 (1.56) | 5.2  (1.98) | 4.9 (1.93) | 2.5  (1.5) | 3.4  (1.68) | 2.6  (1.53) | 0.0 (0.69) | 1.1  (1.13) | 0.0  (0.69) | 2.3  (1.46) | 1.4  b |
| TR+DAP | 3.4  (1.68) | 4.4  (1.85) | 0.7  (1) | 3.2  (1.64) | 0.0  (0.69) | 0.0  (0.69) | 0.0  (0.6) | 0.7  (1) | 1.7  (1.31) | 5.6  (2.03) | 1.3  b |
| T4+DAP | 0.0  (0.69) | 1.0  (1.11) | 0.0 (0.69) | 3.0  (1.61) | 4.1  (1.81) | 0.0  (0.69) | 1.7  (1.3) | 5.1  (1.96) | 0.3  (0.83) | 7.4  (2.24) | 1.3  b |
| Control  (DAP) | 3.0  (1.61) | 1.2  (1.15) | 0.5 (0.92) | 2.4  (1.48) | 2.8  (1.57) | 1.9  (1.36) | 0.0 (0.69 | 1.3  (1.19) | 4.5  (1.87) | 2.5  (1.51) | 1.3  b |
| F ONLY | 6.8  (2.17) | 6.5  (2.14) | 3.9 (1.78) | 17.1 (2.95) | 10.9 (2.56) | 14.1 (2.78) | 3.5  (1.7) | 13.0 (2.71) | 14.4  (2.8) | 20.2  (3.1) | 2.5  a |
| TR ONLY | 16.7 (2.93) | 13.0 (2.71) | 8.8 (2.38) | 17.5 (2.97) | 7.1  (2.21) | 10.1 (2.49) | 2.8 (1.56 | 31.1  (3.5) | 19.3 (3.06) | 21.1  (3.14) | 2.7  a |
| T4 ONLY | 6.5  (2.14) | 10.9 (2.56) | 8.9 (2.39) | 8.0  (2.3) | 16.7 (2.93) | 16.7 (2.93) | 0.9 (1.06) | 29.8 (3.46) | 8.9  (2.39) | 20.2  (3.1) | 2.6a |
| Mean clone | 1.8  bc | 1.9  bc | 1.6  c | 2.2  ab | 1.9  bc | 1.8  bc | 1.1  d | 2.2  ab | 1.9  bc | 2.4  a |  |
| C.V (%) | 36.94 |  |  |  |  |  |  |  |  |  |  |
| LSD (P≤0.05) | |  |  |  |  |  |  |  |  |  |  |
| Treatments | 0.35 |  |  |  |  |  |  |  |  |  |  |
| clones | 0.42 |  |  |  |  |  |  |  |  |  |  |

**Table 4: Effects of treatments on growth of the number of roots after 120 days**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | Tea Clones and number of roots | | | | | | | | | |  |
| **Treatments** | | TRFK  6/8 | TRFK7/9 | TRFK31/8 | TRFK  56/89 | TRK  301/4 | TRFK 303/577 | TRFK 7/3 | TRFK 306/1 | TRFK  597/1 | TRFK  704/2 | mean treats |
| Treatments  F+DAP | | 3 (1.6) | 18(2.9) | 15(2.82) | 4 (1.8) | 4 (1.7) | 2 (1.33) | 1 (0.9) | 1(0.9) | 16(2.91) | 30(3.47) | 2.06d |
| TR+DAP | | 3(1.6) | 16(2.88) | 3(1.6) | 23 (3.2) | 10 (2.5) | 18 (2.9) | 0 (0.6) | 21 (3.13) | 3(1.6) | 31(3.5) | 2.37cd |
| T4+DAP | | 10 (2.5) | 5 (1.9) | 8 (2.31) | 16(2.88) | 27 (3.3) | 13 (2.71) | 4(1.7) | 18 (2.9) | 28(3.41) | 28(3.41) | 2.73b |
| F ONLY | | 17 (2.9) | 18 (2.9) | 28 (3.41) | 19(3.03) | 20 (3.0) | 18 (2.9) | 25 (3.2) | 25 (3.3) | 24(3.24) | 30(3.45) | 3.17a |
| TR ONLY | | 15 (2.8) | 23 (3.2) | 14 (2.76) | 25 (3.3) | 25 (3.3) | 19 (3.03) | 12 (2.6) | 24 (3.26) | 27(3.38) | 26(3.33) | 3.10ab |
| T4 ONLY | | 15 (2.8) | 16 (2.9) | 16(2.88) | 23(3.21) | 28 (3.4) | 13 (2.68) | 6(2.1) | 27 (3.3) | 25(3.29) | 26(3.34) | 3.01ab |
| **Control (DAP only)** | | 21(3.1) | 27 (3.3) | 2(1.29) | 16 (2.88) | 1 (1.1) | 7 (2.21) | 6 (2.1) | 10 (2.45) | 3(1.6) | 12(2.63) | 2.27d |
| Mean clone | | 2.49bc | 2.91ab | 2.44c | 2.90ab | 2.65bc | 2.56bc | 1.95d | 2.78bc | 2.75bc | 3.30a |  |
| C.V (%) | | 27.16 |  |  |  |  |  |  |  |  |  |  |
| LSD (P≤0.05) | | |  |  |  |  |  |  |  |  |  |  |
| Treatments | 0.37 | |  |  |  |  |  |  |  |  |  |  |
| clones | 0.44 | |  |  |  |  |  |  |  |  |  |  |

Table 5 The effects of *T. harzianum* on growth of dry weight after 60 days.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| CLONES | TRFK  6/8 | TRFK 7/9 | TRFK 31/8 | TRFK 56/89 | TRFK 301/4 | TRFK 303/577 | TRFK 7/3 | TRFK 306/1 | TRFK  597/1 | TRFK  704/2 | Mean  treatment |
| Treatments  F ONLY | 0.8 | 0.59 | 0.94 | 0.77 | 0.81 | 0.81 | 0.51 | 0.85 | 1.03 | 0.89 | 0.80abc |
| TR ONLY | 0.91 | 0.72 | 0.86 | 0.67 | 1.48 | 0.75 | 0.33 | 1.07 | 0.84 | 0.65 | 0.83ab |
| T4 ONLY | 1.16 | 0.46 | 0.8 | 0.61 | 0.86 | 0.72 | 0.29 | 1.37 | 0.71 | 0.63 | 0.76bc |
| F+DAP | 0.7 | 0.71 | 1.07 | 0.64 | 1.36 | 0.98 | 0.43 | 1.14 | 0.77 | 0.76 | 0.86ab |
| TR+DAP | 0.81 | 0.7 | 0.88 | 0.63 | 1.52 | 0.94 | 0.46 | 0.97 | 0.96 | 0.87 | 0.88a |
| T4+DAP | 0.61 | 0.7 | 0.79 | 0.57 | 1.01 | 0.72 | 0.42 | 1.25 | 0.93 | 0.71 | 0.77abc |
| Control  (DAP) | 0.62 | 0.56 | 0.75 | 0.88 | 0.95 | 0.86 | 0.26 | 0.87 | 0.58 | 0.63 | 0.70c |
| Mean clone | 0.80  bcd | 0.64  e | 0.87  b | 0.69  de | 1.14  a | 0.83  bc | 0.38f | 1.07  a | 0.83  bc | 0.74  cde |  |
| C.V (%) | 27.32 |  |  |  |  |  |  |  |  |  |  |
| LSD (P≤0.05) |  |  |  |  |  |  |  |  |  |  |  |
| Treatments | 0.112 |  |  |  |  |  |  |  |  |  |  |
| clones | 0.133 |  |  |  |  |  |  |  |  |  |  |

### Table 6: Effects of treatments on root biomass after 120 days

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Tea clones and root biomass (Dry weight (g)** | | | | | | | | | |  |
| **Treatments** | TRFK 6/8 | TRFK 7/9 | TRFK 31/8 | TRFK 56/89 | TRFK 301/4 | TRFK 303/577 | TRFK 7/3 | TRFK 306/1 | TRFK 597/1 | TRFK 704/2 | mean treats |
| Treatment  FONLY | 1.17 | 1.49 | 0.79 | 1.81 | 2.37 | 1.18 | 1.16 | 1.71 | 1.51 | 1.14 | 1.4ab |
| TRONLY | 0.97 | 0.69 | 1.7 | 1.78 | 1.4 | 1.09 | 0.74 | 1.79 | 1.48 | 1.3 | 1.3bcd |
| T4 ONLY | 0.96 | 1.44 | 1.68 | 1.63 | 1.82 | 1.31 | 0.8 | 1.2 | 1.43 | 1.29 | 1.4ab |
| F+DAP | 0.84 | 1.46 | 1.65 | 1.67 | 1.43 | 0.69 | 0.61 | 1.63 | 1.63 | 3.49 | 1.5a |
| TR+DAP | 0.86 | 0.75 | 2.54 | 1.72 | 1.84 | 2.16 | 0.56 | 1.22 | 1.49 | 1.86 | 1.5a |
| T4+DAP | 0.91 | 0.9 | 1.81 | 0.94 | 1.95 | 1.28 | 0.49 | 1.34 | 1.85 | 1.25 | 1.3cd |
| Control  (DAP) | 0.8 | 0.7 | 0.789 | 1.24 | 1.65 | 0.97 | 0.54 | 1.64 | 1.22 | 1.43 | 1.1d |
| Mean clone | 0.9d | 1.1c | 1.6b | 1.5b | 1.8a | 1.2c | 0.7e | 1.5b | 1.5b | 1.7ab |  |
| C.V (%) | 21.7 |  |  |  |  |  |  |  |  |  |  |
| Treatments | 0.15 |  |  |  |  |  |  |  |  |  |  |
| Clones | 0.18 |  |  |  |  |  |  |  |  |  |  |

# CONCLUSIONS AND RECOMMENDATIONS

Treatments; F ONLY; TR ONLY; T4+DAP and T4 ONLY showed the highest development of roots. Treatments; TR ONLY; T4 ONLY; F ONLY; T4+DAP showed the highest number of roots. Treatments; F ONLY; T4 ONLY; TR+DAP; F+DAP showed the highest dry weight. Clones; TRFK 56/89; TRFK 7/9; TRFK 704/2 produced the highest root length, Clones; TRFK 704/2; TRFK 7/9; TRFK 56/89 produced the highest number of roots, Clone TRFK 301/4; TRFK 704/2;597/1; 306/1;56/89 producing the highest mean dry weight. *Trichoderma spp.* F, TR, T4 enhanced growth of tea clones when applied singly or in combination with DAP fertilizers. Further research should be carried out on clone TRFK 7/3 whose overall growth was least enhanced by all the treatment compared to the other clones. Further research to be carried out on the effects of the other Trichoderma spp. isolates on growth of tea cuttings and other tea clones not covered by the research.

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