

Induction of fine roots in *Leucaena leucocephala* using *Agrobacterium rhizogenes***Mohammed Saifuddin, Divya Mariam Chandy, Normaniza Osman, Norzulaani Khalid****Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*****Corresponding author: saifuddin@siswa.um.edu.my****Abstract**

Leucaena leucocephala has been previously documented to be a potential slope plant due to its prominent tap root system. However, this plant lacks of fine roots which arguably contribute to reinforce the soil. Thus, this experiment was aimed to induce fine roots of *L. leucocephala* by infecting *Agrobacterium rhizogenes*. The *A. rhizogenes* was cultured under optimum nutrient conditions and injected into the hypocotyls of *L. leucocephala*, seedlings. The infected seedlings were transplanted into PVC pipes filled with garden soil. The plant infected by *A. rhizogenes* having 1.09×10^9 cfu/mL (T1) concentrations showed the highest root length of 1018 cm (83.2% fine roots) as compared to the control. The increased of overall root volume provides a greater surface area which increased (55%) the Water Absorption Capacity (WAC) significantly. The number of fine roots and root tips were increased by 106 and 261%, respectively by T1 treatment. The photosynthetic and transpiration rate also exhibited significant increment (98% at $p < 0.05$) in T1 than the control plants. The above findings have referred a better understanding of the effects of *A. rhizogenes* infection on hairy root initiation, WAC and physiological aspects of the *L. leucocephala*, potential slope plant. Hence, it is suggested that *A. rhizogenes* infection can be applied to initiate extensive fine roots, enhancing the root system of potential slope plants.

Keywords: Fine roots, *Agrobacterium rhizogenes*, photosynthetic rate, transpiration rate and Water Absorption Capacity.

Abbreviations: WAC Water Absorption Capacity; OD Optical Density; PVC Polyvinyl Chloride; RLD Root Length Density.

Introduction

In soil bioengineering techniques, the use of vegetation is highly recommended as a structural element to stabilize natural and man-made slope due to their eco-friendly solutions. The root system of a plant functions and contributes to the slope stability in various ways including reducing the water level of soil, providing essential nutrients to plant growth, plant anchorage, storage of water and its photosynthetic assimilates (Baets et al., 2008; Danjon et al., 2008; Normaniza and Barakbah, 2006; Pierret, 2007). Root growth induces many changes in soil physical and chemical properties which indirectly influenced the soil reinforcement too (Wang et al., 2003). Looking at a physical aspect, the root, while providing anchorage for the plant, will also hold the soil together by enhancing its soil cohesion. In addition to that, root systems can prevent soil from storing excess water which could otherwise lead to possible slope failures (Bengough et al., 2006; Cammeraat et al., 2005; Normaniza and Barakbah, 2006; Morgan, 2007). As a plant absorbs water, soil will become less saturated which will result in the slope of being drier, thus reducing the possibility of slope failure. As of such, root establishment is to be encouraged on slope sites to promote slope stability. The reinforcing and water absorption effects of plant roots on the stability of slope are attributed to the increase of shear strength of the soil which is increased by anchoring a soil layer and by forming a binding network within that layer (Mattia et al., 2005). *Leucaena leucocephala* is a versatile leguminous plant which has been determined as a potential slope plant (Parera, 1982; Normaniza et al., 2008). *L. leucocephala* is supported by deep tap and lateral roots. This plant extensively forms nodules to perform symbiotic process. However, the presence of hairy root is few. It is well documented that hairy or fine

roots are important for strengthening soil anchorage and possibly have higher water absorption capacity (WAC) than thick roots which are needed as a potential slope plants. Fine roots also have high capacity to increase soil cohesion, which can reduce land-slide risks. Ample research has been carried out on the genetic transformation of the *A. rhizogenes* with regards to gene transfer and metabolite improvement but hardly any on the morphological properties of these hairy or fine roots and its effects on slope stability. *A. rhizogenes* is a soil-borne plant pathogen which is responsible for adventitious hairy root formation at the site of infection (Barbara et al., 2008). Hairy root is a phase during which there is abnormal development of fine fibrous roots. *Agrobacterium* is attracted to host across chemical gradient of phenolic compounds released by injured plant cells, specifically acetosyringone which had been reported to increase *Agrobacterium*-mediated transformation frequencies in a number of plants (Ionkova, 2007). In order to succeed in establishing a hairy roots culture system for a certain plant species, several essential conditions should be taken into considerations including the bacterial strain of *A. rhizogenes*, appropriate explants and a proper antibiotic to eliminate redundant bacteria after co-cultivation (Hu and Du, 2006). But the documentation on tropical woody species is limited. And the influence of *A. rhizogenes* on plant physiology, root initiation and WAC are not well explained and understood. The objectives of this study are to induce fine or hairy roots using biotechnological technique, infecting *A. rhizogenes* on the *L. leucocephala* seedlings and to assess the plant physiology and root profile of the infected plants to see its implications as a potential slope plant.

Table 1. Root profile of *L. leucocephala* after three months of growth.

Treatments	Control	T1	T2	T3	T4
Hairy roots (%)	40.3 ± 1.5c	83.2 ± 1.3a	70.6 ± 0.6b	80.8 ± 0.2a	80.3 ± 0.3a
Number of tips	277 ± 14.5d	1001 ± 128a	867 ± 13.2b	412 ± 11.5c	478 ± 68c
Average diameter (mm)	0.731 ± 0.02a	0.46 ± 0.03b	0.409 ± 0.04c	0.49 ± 0.04b	0.49 ± 0.03b

T1, T2, T3 and T4 are different concentrated *A. rhizogenes* culture (Fig. 13).

Table 2. Water Absorption Capacity and related parameters.

Treatment	<i>E</i>	WAR	WAC
Control	4677±217de	4773	11.23
T1	17383±606a	17738	17.42
T2	12140±59b	12387	12.73
T3	6236±60c	6363	14.63
T4	5378±52cd	5487	13.03

Where, *E* = transpiration rate (L H₂O/plant/day); WAR (transpiration rate × 100)/98 (L H₂O/plant/day); WAC = WAR/total root length (L H₂O/cm root/day). Means (data are means ± standard error) with different letters were significantly different (p < 0.05, ANOVA).

Table 3. There is no significant correlation between WAC and the photosynthetic rate, stomatal conductance and transpiration rate

Correlation	R	R ²
WAC vs photosynthetic rate	0.064	0.004
WAC vs stomatal conductance	0.158	0.024
WAC vs transpiration rate	0.155	0.024

Results

Physiological aspects of *L. leucocephala*

The photosynthetic rate, stomatal conductance and transpiration rate showed a significant difference among the treatments. T1 showed the highest value in photosynthetic rate (Fig. 1), stomatal conductance (Fig. 2) and transpiration rate (Fig. 3). Higher photosynthetic rate of T1 plants indicate that they are more efficient in utilizing light energy to increase in plant vigor and growth. In addition, the transpiration rate was measured for 12 hours, i.e. diurnally and T1 treatment showed the highest value. Transpiration rates of all treatments were low in the morning and late morning but high at midday (Fig. 4). This is a typical diurnal pattern found in all treatments. But, among the treatments, T1 treatment had the highest transpiration rate at midday which was nearly four times higher than control. T2, T3 and T4 showed the diurnal transpiration rate of 0.022, 0.025 and 0.025 mmol m⁻² s⁻¹, respectively (Fig 4). Higher transpiration rates can, arguably, be taken to mean extensive rooting system. As a result, the higher the diurnal pattern, transpiration rates, of T1 refers the higher efficiency in utilizing the leaves for enhancing evaporation of water and are more likely to increase WAC, a characteristic essential for a slope colonizer.

Root profile analysis

The root length and volume of T1, high concentrated infection of *A. rhizogenes*, had reached 1018 (cm) and 4.7 cm³, respectively, in which the performance was outstanding, compared to other concentrated infections. It was observed that there was an increment of root length and volume which was proportionate to the concentration of the *A. rhizogenes*, culture solution (Fig. 5, Fig. 6 and Fig. 7). The percentage of hairy roots, number of tips and average root diameter of *L. leucocephala* showed significant difference among the treatments (Table 1). T1 showed the highest percentage of hairy roots and number of root tips. T2, T3 and T4, nonetheless, showed a higher value of these parameters than

the control. These hairy roots are considered as roots with a lower diameter denoted as also fine roots. Root pattern of the infected *L. leucocephala* plants after three months of growth were showed in Figure 5.

Water absorption capacity (WAC) of plants and correlation among WAC and physiological and root profiles

In the case of WAC of *L. leucocephala*, T1 showed a spectacular increase of 55% from the control treatment (Table 2). Nonetheless, T2, T3 and T4 showed also higher value than that of the control treatment. Water absorption by roots and transpiration by stomata are continuous process and most of the cases are proportional to each other. But it varied by the weather conditions and also depended on various treatments (Endres et al., 2010). It was envisaged that the physiological phenomena of plant was more related to the water availability, not depending on the water absorption capacity of the plants. Previous Figures (1, 2, 3 and 4) showed that the photosynthetic rate, stomatal conductance and transpiration rate was higher in treated plants than the untreated plants. But in Table 3, there was no correlation was found between the WAC and the photosynthetic rate, stomatal conductance and transpiration rate. This was due to WAC might not be strongly dependent on photosynthetic rate, stomatal conductance and transpiration rate. Though, the process of water absorption by root significantly contributed to most physiological process and plant development. Whereas, there is a positive correlation was observed between plant WAC and root volume (Figure 8). The higher the underground root volume refers the higher WAC of plants. It is well documented that water in the roots is pulled through the plant by transpiration. Therefore, an increase root volume is greatly beneficial in absorbing the excess soil water and removed them out to the atmosphere via transpiration. As a result, removed excessive water would lead in drying the slope and stability of the slope. Tognetti et al. (2009) also described that higher root biomass would be

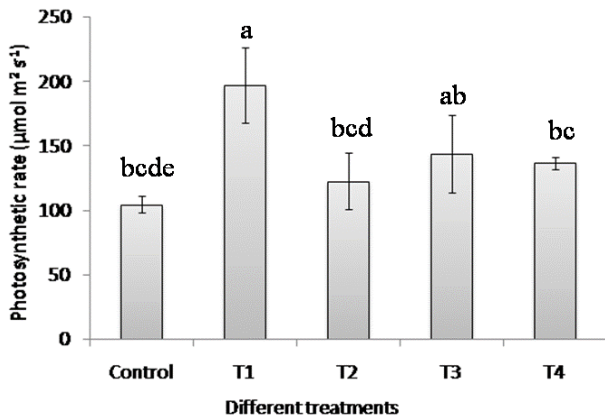


Fig 1. Photosynthetic rate of all treatments after three months of growth. Means (data are means \pm standard error) with different letters were significantly different ($p < 0.05$, ANOVA).

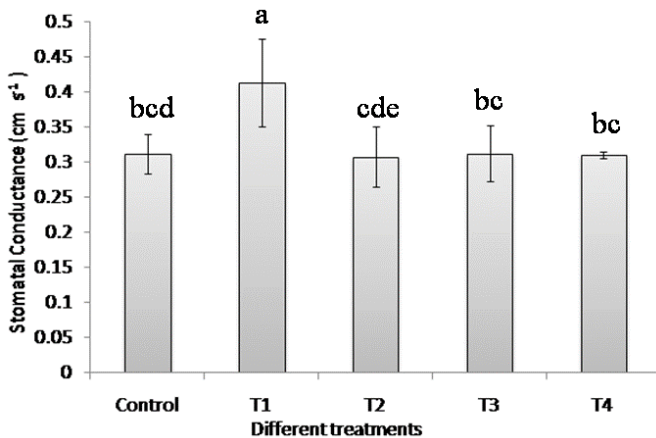


Fig 2. The stomatal conductance of all treatments after three months of growth. Means (data are means \pm standard error) with different letters were significantly different ($p < 0.05$, ANOVA).

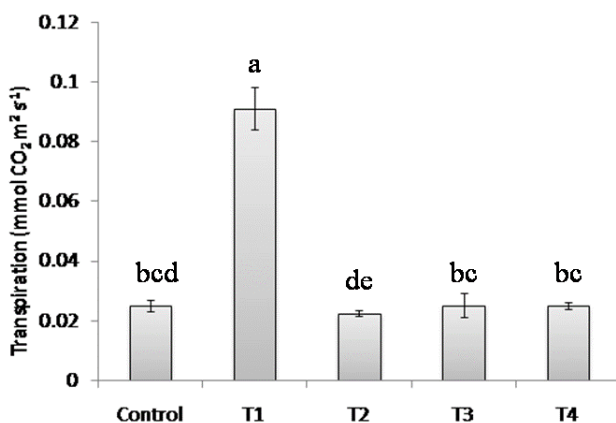


Fig 3. Transpiration rate of all different treatments after three months of growth. Means (data are means \pm standard error) with different letters were significantly different ($p < 0.05$, ANOVA).

advantageous to absorb extra soil water and to enhance water movement from soil to plant body.

Discussion

The differences in fine roots, root tips and root average diameter among the different concentrated injected plants were exhibited in Table 1. Higher number of hairy roots and root tips were observed in the injected plants. It can be assumed that higher concentrated *Agrobacterium rhizogenes* have the remarkable capacity to transmit their T-DNA gene into the plants that allowed producing more hairy roots and number of root tips. Additionally, in case of average root diameter, different values were observed for different concentrations of *A. rhizogenes*. The results showed that lower diameter root seemed to be related with injected plants. Overall findings provide strong evidence that the variation of *A. rhizogenes* concentration is related in root hairy root production and variation in root system. After an inoculation period of 36 hours, the infection of *A. rhizogenes* has increased the hydrological role of *L. leucocephala* by increasing the WAC by 55% in T1, as compared to the control treatment. T2 and T3 also showed an enhancement of the root profile of the *L. leucocephala*. T1 proved to be the best treatment with a concentration of 1.09×10^9 cfu/mL. Therefore, infection by undiluted cultured bacteria is the best way to increase root length and volume. T2 and T3 also showed an enhancement of the root profile, which presumably is related to the increased root volume leading to increase water absorption of the *L. leucocephala*. The increment of overall root volume provides a greater surface area to cover additional soil for water absorption. Eduardo et al. (2008) demonstrated that hairy roots are capable to absorb relatively more water and minerals due to high root surface area. The increment of root length would also increase water absorption as well as provide better anchorage systems. In this study however, *A. rhizogenes* was successfully increase the number of root tips and the physiological activity of the plant. These improvements are assumed for the increment of total root profiles to absorb additional water and minerals. Therefore, utilization of additional water resources could cause corresponding changes in plant physiological factors also such as photosynthesis and transpiration. The enhancement of physiological activity of *L. leucocephala* was arguably due to the different root profiles. It was reported that the rate of transpiration is strongly related to the stomatal conductance (Endres et al., 2010). Thus stomata are required to maintain a water-photosynthesis syndrome, regulating the transpiration rate and the photosynthetic rate (Purohit, 2003). Most studies indicated that in transpiration process plant uses about 90% of water. Total root length per unit ground area is often considered to be directly related to the amount and rate of water uptake (Normaniza and Barakbah, 2006). The results were arguably attributed to high root biomass and composition existence above the ground, which most likely resulted in high water absorption by root and soil water loss through transpiration. The high capacity of water absorption through a plant would cause less water-saturated soils, causing the slope to become drier. This reduces the possibility of slope failure. It was discovered by Normaniza et al. (2009) that the root reinforcement is much higher when the soil is less saturated with water. *L. leucocephala* also showed a relatively high WAC as compared to other potential slope plants that have been previously examined including *Vertiver* sp., *Justica* sp., *Bixa* sp., *Bauhinia* sp., and grasses (Normaniza, 1998; Normaniza et al., 2008). High WAC of

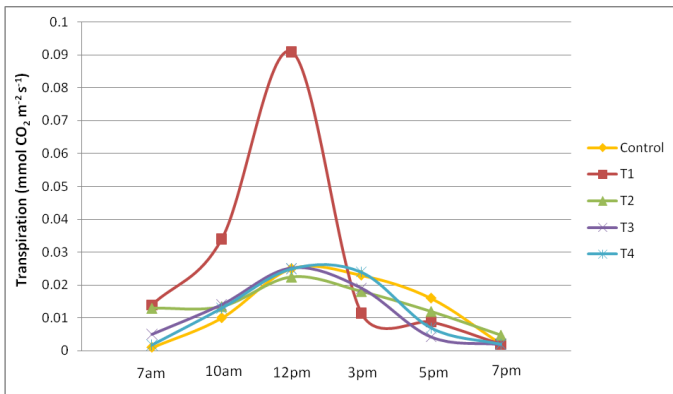


Fig 4. Diurnal transpiration patterns after three months of growth.

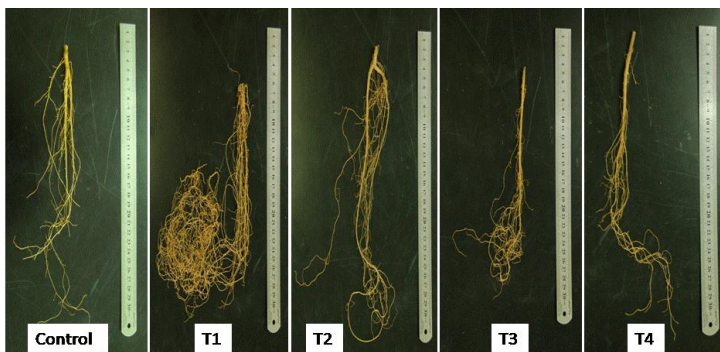


Fig 5. Root profile of the infected *L. leucocephala* plants after three months of growth.

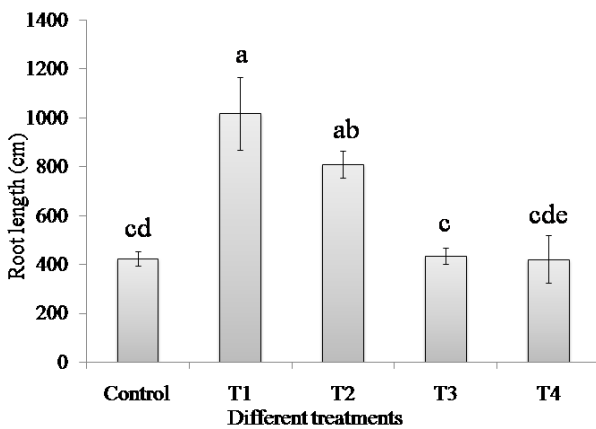


Fig 6. Root length (cm) in different treatments. Means (data are means \pm standard error) with different letters were significantly different ($p < 0.05$, ANOVA).

plant resulted less saturated soil by water. Therefore, the presence of more fine roots reduce the possibility of slope failure. Pollen (2007) formed a model where showed that the root reinforcement was lower when the soil moisture content was higher. Due to this, the root reinforcement is most likely to be minimal when the soil is saturated with water. In addition to that, there was a significant correlation between root length density, soil water content and slope stability, which was showed by Normaniza and Barakbah (2006). It was also suggested that root length density and soil water level could be used as an indicator of slope stability and to predict the probability of slope failure. Therefore, extensive root profiles of a slope plants can strongly help or refer in the

strengthening the slope *via* its capability to absorb sufficient water, in turn lowering the risk of landslides and erosion. Another previous study showed a positive correlation between the shear strength and the Root Length Density (RLD). The value of RLD was usually considered by measuring the root length in relation to the volume of soil. This would substantially help in soil reinforcement at the surface level, thus reducing surface erosion. Studies by O'Loughlin (1984) indicated that the shear strength provided by fine roots of 0.0 mm to 2.0 mm contributes the most in soil reinforcement. Consequently, there is a positive correlation observed between WAC and root volume (Fig. 8), implying that the WAC would be higher if the root volume is higher. Whereas, hairy roots (%) and root diameter are negatively correlated (Fig. 9). Number of hairy roots increases with decreasing root average diameter. In addition, the results also showed that the percentage of fine roots seemed to moderately correlated with WAC ($R^2 = 0.54$, Fig. 10), possibility due to the hairy roots property and the increment in root-soil bond. Therefore, these findings have led to the proposal of root-water relation and that was, lower diameter root growth promoted the hairy roots growth and water uptake. Consequently, an increase of lower diameter root biomass e.g. hairy roots (%) is greatly beneficial in absorbing the excess soil water and removed them out to the atmosphere via transpiration (Kendy et al., 2003). As a result, removed excessive water would lead in drying the slope and stability of slope. In addition, the higher the root length referred the higher number of root tips and root volume. As a result, increased additional root system by treatment (T1) would lead in increasing root soil interaction in slope and stability of the slope.

Materials and methods

Agrobacterium rhizogenes culture, growth and inoculation

A wire loop was sterilized by dipping it into 70% ethanol and heated till red. Once the loop cooled, it was dipped into glycerol stock containing *Agrobacterium rhizogenes* wild type strain 8196 and quickly streaked over a prepared LB Agar in a Petri dish. The Petri dish was then sealed with parafilm and incubated in a dark place at room temperature. This was repeated to obtain more culture stock. After 2 days, the Petri dish showed single colony of bacteria. Part of the bacterial colony from the Petri dish was then transferred and mixed with LB broth contained in the universal bottle. This process again was repeated until sufficient cultures were obtained (Fig. 11). The tightly sealed universal bottles were then placed on a rotary shaker, at the speed of 250 rpm in the dark and at room temperature. After 48 hours, the bacteria culture turned cloudy and was then stored at 4°C until needed.

Time of infection and serial dilution of the *A. rhizogenes*

As the bacteria multiplies in the nutrient broth under its optimum conditions, the Optical Density (OD) increases. The growth curve of the *A. rhizogenes* was plotted to determine the optimum time for infection (Fig. 12). The curve obtained from plotting the growth curve which is divided into three phases namely the lag-phase, log-phase and stationary phase as illustrated in Figure 12. At the hour of 36, the OD reads at 1.83×10^{-6} nm, assuming that the cell density of 1 nm OD was equivalent to a million (1×10^6) colony forming unit per milliliter (cfu/mL). From this, it was derived that at 36 hours, the assumed cell density was 1.09×10^9 cfu/mL. This

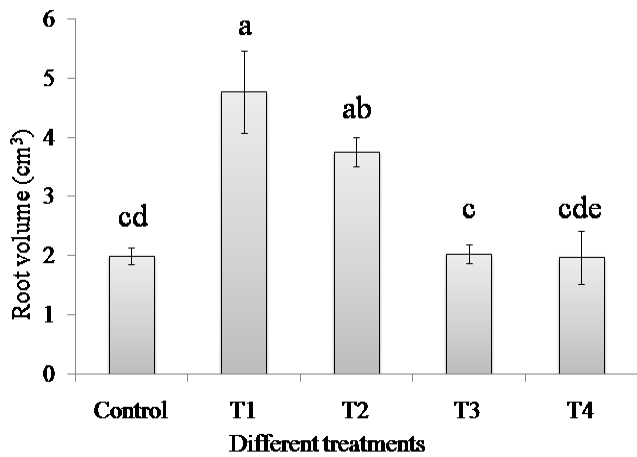


Fig 7. Root volume (cm³) in different treatments. Means (data are means ± standard error) with different letters were significantly different ($p < 0.05$, ANOVA).

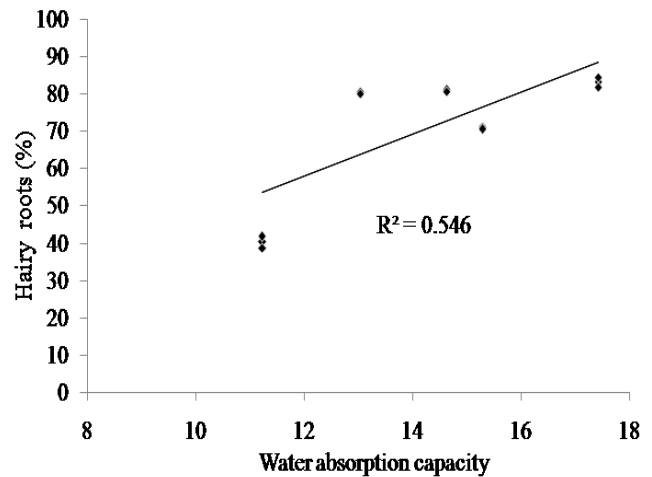


Fig 10. A weak relationship observed between water absorption capacity (WAC) and hairy roots (%).

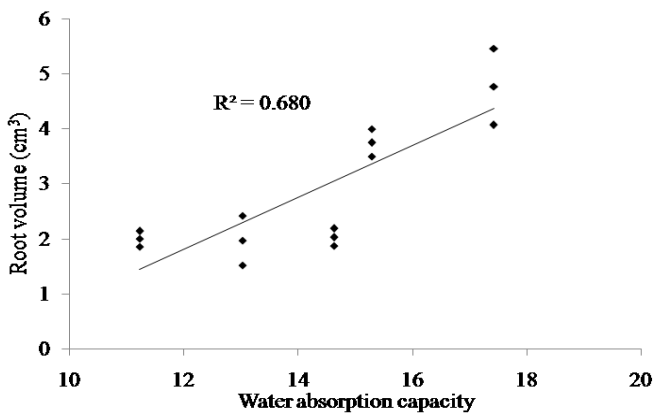


Fig 8. A strong relationship between water absorption capacity (WAC) and root volume (cm³).

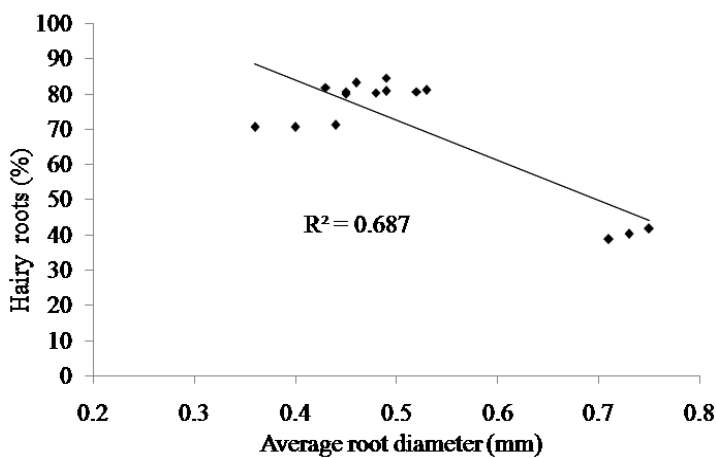


Fig 9. Relationship between hairy roots (%) and average root diameter (mm)

concentration was regarded as T1 treatment. Apart from the control (no infection) serial dilution was carried out to ascertain the treatments according to concentrations 10^0 , 10^{-1} , 10^{-2} and 10^{-3} of the original culture. This was done by pipetting 100 μ L of bacterial culture into 9.9 mL sterile distilled water (Fig. 13). This technique was repeated to achieve dilutions at 10^{-1} , 10^{-2} and 10^{-3} .

Ex vitro approach: seed pre-treatment and planting

L. leucocephala seeds were soaked in warm water overnight for imbibitions to take place. Seeds were then blotted dry and mixed with 3 g of *Rhizobium* compost culture. Water (2 mL) was added to increase surface interaction between *Rhizobium* and seed. The rock phosphate (3 g) is mixed into the mixture and is left to soak for 10 minutes to ease the rooting operation. The seeds were then sown into a 30 cm by 50 cm basin filled with garden soil. The seeds were watered once every day and allowed to grow in the glasshouse.

Infecting Ex vitro

Using a 1mL Terumo® Syringes with needle, the *A. rhizogenes* cultures, according to its treatments, were injected into the hypocotyls of the seedlings (Cao et al., 2009) (Fig. 3Y). The infected plants were then transferred into polyvinyl chloride (PVC) pipes filled with garden soil and lined with plastic bags. The plants were arranged in a completely randomized design (CRD), with 25 cm row distances and 25 cm plant to plant distances under glasshouse condition (temperature 21-32°C, maximum PAR 2100 μ E m⁻² s⁻¹ and relative humidity of 60-90%). Three replicates were done for each concentration. Plants were watered daily to avoid water stress. Weeds that grew in the PVC-pipes during this period were removed by hand.

Photosynthesis, transpiration rate and stomatal conductance

The photosynthetic rate, transpiration rate and the stomatal conductance of *L. leucocephala* in all treatments was measured using the Portable Photosynthesis System (Li-COR, CIRAS-1, USA) after three months. The diurnal

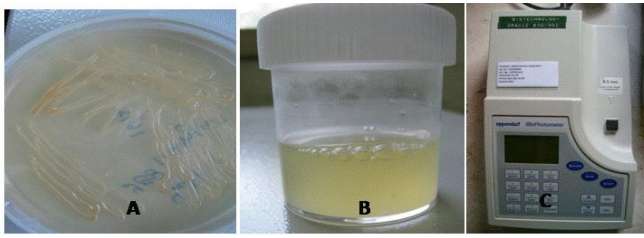


Fig 11. A) *A. rhizogenes* bacterial colony in petri dish, B) Cloudy bacterial culture after 48 hours of incubation, C) Eppendorf® Biophotometer used to measure Optical Density.

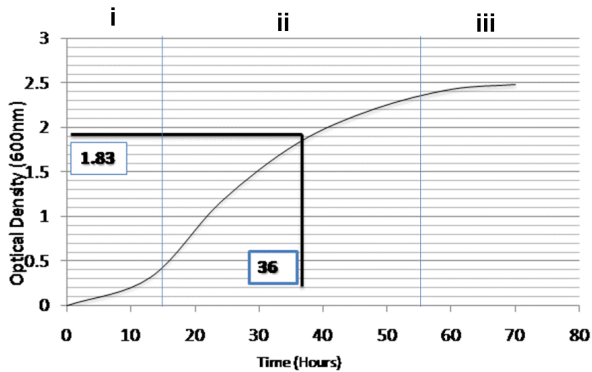


Fig 12. Growth curve of *A. rhizogenes*. Where, (i) lag-phase, (ii) log-phase and (iii) stationary phase. The best time for infection onto the *L. leucocephala* was found to be at hour 36, which is at mid-log point.

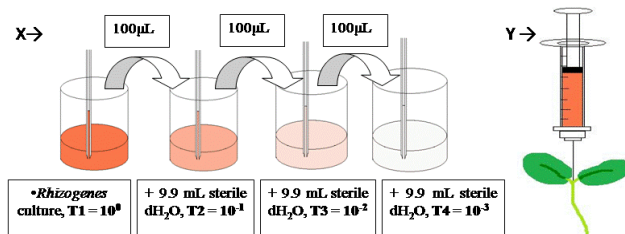


Fig 13. “X” is illustration of serial dilution process and “Y” is injection of *A. rhizogenes* into the hypocotyl of *L. leucocephala*

transpiration rate was deliberated from calculating the area under the curve of each treatment.

Root Profile

The root length and volume of all the different treatments of *L. leucocephala* were determined by scanning and using the WinRHIZO Pro Software after three months. This software was also used to find the percentage of hairy roots, number of root tips and the average diameter of the root.

Water Absorbtion Capacity (WAC)

The WAC is a formula based on the Baker’s theory (Baker, 1984). According to this theory, 98% of the water absorbed

by roots is transpired into the atmosphere. This statement leads to the formula as follows:

$$\text{WAR} = \text{Water Absorption Rate by root/day} \\ (\text{L H}_2\text{O/plant/day}) = E^{\theta} \times 100/98$$

Where; E^{θ} = Diurnal transpiration rate (L $\text{H}_2\text{O/plant/day}$)

$$= Y \times A \times \text{Molecule weight of water} \times \text{time} \\ = \Sigma E (\text{mmol m}^{-2} \text{s}^{-1}) \times \text{leaf area (m}^2) \times 0.000018 \text{ Kg} \times 12 \times 60 \times 60 \text{ s}$$

WAC = Water Absorption Capacity by root

$$(\text{L H}_2\text{O/cm root/day}) = \text{WAR}/\text{total root length}$$

Statistical Analysis

Statistical analysis (experimental design was CRD having three replications per treatment) was performed using SPSS software. LSD ($p=0.05$) was calculated using the error mean squares of the analysis of variance. The correlation test between the parameters studied was analyzed using Microsoft Excel.

Conclusions

In conclusion, present study was conducted to investigate the hairy root transformation process, plant physiological and root profiles affecting *A. rhizogenes*-mediated transformation in *L. leucocephala*. Higher concentration, 1.09×10^9 cfu/mL (T1), was found to be superior for genetic transformation mediated by *A. rhizogenes*. *A. rhizogenes* has successfully enhanced the root system with hairy roots. The increment of root profile was strongly increased the Water Absorption Capacity of plants, presumably due to the enhancements of root volume and transpiration rate. The increased hairy roots resulted in the decreased root diameter also. Genetic transformation methodology can possibly be a good tool for improving the quality of a plant’s root profile to boost its physiological (photosynthesis and transpiration) and mechanical (root system) capacity as a potential slope plant. This study is still in premature, further studies should be required to better understand how physiological parameters are related to each other and how *A. rhizogenes* influence the overall physiological functions on a potential slope plant.

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