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Full Length Research Paper

Isolation of indigenous rhizobium strain from *A. confusa* for re-inoculating *A. confusa* seedlings

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Reforestation of native *Acacia confusa* Merr. on landslide areas in Taiwan is important for agroforestry and soil conservation. To ensure high survival and growth vigor, *A. confusa* seedlings must develop a strong root system. Inoculating of acacia with symbiotic nitrogen-fixing bacteria (NFB) may ameliorate the problems associated with soil nutrient deficiency on landslide sites. In this study, under plastic house condition, a NFB was isolated from the root nodules of native *A. confusa* and identified as *Bradyrhizobium elkanii*, and its effects on growth, root system morphology and pullout resistance of acacia seedlings were investigated. Our results revealed that the growth of inoculated seedlings is significantly more vigor than that of the noninoculated controls. The enhancements in height, tap root length, shoot biomass and root biomass were 40, 100, 140 and 130%, respectively. Also, inoculated seedlings had significantly longer total root length (150%), larger external root surface area (130%), larger root volume (70%), and more root tip number (60%) than the controls. Moreover, the inoculated seedlings developed significantly stronger root functional traits, that is, root density (130%), root length density (60%) and specific root length (60%), than the controls. Consistently, the root pullout resistance of inoculated seedlings was significantly higher than that of the noninoculated ones. These results demonstrate that *B. elkanii* is an effective nitrogen-fixing bacterium capable of enhancing growth, root development and pullout resistance of *A. confusa*.

Key words: Fabaceae, inoculation, nodules, pullout resistance, root morphology.

INTRODUCTION

Acacia confusa (*Acacia confusa* Merr.), belonging to the family of Fabaceae, subfamily Mimosoideae, is an endemic nitrogen-fixing hardwood tree, widespread throughout the island of Taiwan (Huang and Ohashi, 1977). *Acacia confusa* has high potential for agroforestry, lumber production and landslide prevention, and reforestation. *Acacia* species can symbiose with nitrogen-fixing microorganisms (that is, *Rhizobium* spp.,

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Bradyrhizobium spp., *Azorhizobium* spp., and *Mesorhizobium* sp.) and form nitrogen-fixing nodules, which can fix nitrogen from air and supply nitrogen nutrient to trees for growth and development (Ferro et al., 2000; Zerhari et al., 2000; Dumroese et al., 2009; Ceccon et al., 2011; Diouf et al., 2015; Pereyra et al., 2015). Several studies have demonstrated that inoculation of seedlings (*Oryza sativa*, *Glycine max* and *Zea mays*) with rhizobial strains results in the change of root morphology, that is, increases in nodules, lateral roots, root hairs, root surface area, and total root length (Huang and Ladha, 1997; Ikeda, 1999; Souleimanov et al., 2002).

Morphological shapes of tree root systems have been categorized into three shapes, that is, taproot, heart root and plate root (Wilde, 1958). Yen (1987) classified the patterns of root growth in trees into five types, that is, H-type, R-type, VH-type, V-type and M-type. Styczen and Morgan (1995) classified root systems into three types, that is, H-type with horizontal lateral roots, VH-type with deep taproots and M-type with profusely branching roots. Trees generally develop two main classes of roots, the long taproots ensuring tree anchorage and the short lateral roots (Stokes et al., 2008). Trees with heart root and taproot systems are more resistant to uprooting than plate root systems (Dupuy et al., 2005). Wu et al. (2004) suggested that taprooted trees could be better for slope stability due to the increased slope factor of safety. The type of tree roots changes root system morphology, which in turn affects their biomechanical properties (Burylo et al., 2012). Previous studies on root distribution, root system morphology and mechanical properties of *Acacia* species were concentrated on *A. mangium*. In *A. mangium*, about 35% of its roots are distributed in the top 10 cm layer, and about 90% of its roots is in the fine root class (d < 2 mm) (Lateh et al., 2014; Avani et al., 2014). The pullout resistance of *A. mangium* is much influenced by the root than the shoot (Normaniza et al., 2011; Lateh et al., 2015). However, rhizobial symbiosis and its effect on growth, root system morphology and root biomechanics of *A. confusa* have not been investigated. The aim of the study was to (1) isolate the indigenous rhizobium strain from *A. confusa* for re-inoculating *A. confusa* seedlings, and (2) investigate the effects of the rhizobium inoculation on growth, root system morphology and pullout resistance of seedlings in order to provide strategy for landslide reforestation and soil conservation.

MATERIALS AND METHODS

Sample collection

In September 2014, an elite tree in the natural forest of *A. confusa* located at Chiayi, Taiwan (120°29'09''E, 23°28'06''N) was selected according to height, diameter at breast height (DBH), straightness, and absence of trunk defects. Root nodules were collected from roots at 5 to 20 cm depth of soil stratum, put in polyethylene bags and stored at 4°C until rhizobial strain isolation. Seeds were also collected from the same tree.

Rhizobial strain isolation and purification

Root nodules were cleaned with ultrasonic cleaner, surface sterilized twice with 75% ethanol for 5 min, rinsed by sterile water for 3 times, then sterilized 3 times with 10% sodium hypoclorite for 5 min and rinsed by sterile water for 4 times. The endophytic bacteria in nodules were isolated and purified with streak-plate method on YEMA medium (Usharani et al., 2014). The purified strain was designated as AC1.

DNA extraction, sequencing, and phylogenetic analysis

Bacterial genomic DNA extraction kit (GenElute NA2100, Sigma-Aldrich, St. Louis, MO, USA) was used for total DNA extraction, and subsequently subjected to 1.2% agarose gel electrophoresis. The spectrophotometric absorption values of the nucleic acids were used for concentration measurement. The primers used for PCR of 16S rRNA were forward primer U1 (5'-ACGCGTCGACAGAGTTTGATCCTGGCT-3'), U1R (5'-GGACTACCAGGGTATCTAAT-3') (Relman, 1993), and reverse primer 27f (5'-AGAGTTTGAC MTGGCTCAG-3'), U1492R (5'-GGT TAC CTT GTT ACG ACTT-3') (Weisburg et al., 1991). After PCR reaction, 2.5 µl amplification samples was loaded to 1.2% agarose gel for electrophoresis at 100 V, and the specific PCR products were sequenced (Tri-I Biotech, Taichung, Taiwan). The sequences were logged into NCBI to access GenBank for sequence similarity and homology study of the 16S rRNA gene sequences. The phylogenetic relationships were analyzed by Molecular Evolutionary Genetics Analysis (MEGA, Bioinformatics, Arizona State Univ., Tempe, USA). Strains with high similarity in sequence were chosen for similarity analysis.

Establishment of seedlings

After surface cleaning with tap water, seeds of *A. confusa* were sterilized with 10% sodium hypochlorite (NaOCl) solution, and pretreated with hot water (80°C) for 10 min, rinsed three times with sterilized distilled water, and then germinated in autoclaved peat moss and vermiculite mixtures (1:1, v/v) in the spring. The containers used for transplanting were wooden plant culture boxes (*l*×*w*×*h*, 30 cm × 30 cm × 60 cm) (Figure 2). Two weeks before transplanting, the containers were sterilized with 10% sodium hypochlorite solution, and sandy loam soils were sterilized with steam and then fumigated with 20 g Basamid granular soil fumigant (Engage Agro Corp., ON, Canada) for 2 days. The sterilized soil was then used to fill containers. When seedlings attained 5 cm in height, they were transplanted to the containers one plant in each container, and watered every morning. Thirty-two seedlings in containers were randomly arranged into two separate plastic houses.

Inoculation test

One week after transplanting, 16 seedlings in one plastic house were inoculated with the isolated rhizobial strain AC1 of *B. elkanii*. A 5 ml suspension of the strain (1.5×10^8 cfu/ml) was applied into four holes around the seedling. The same inoculation was repeated two weeks later in order to ensure high colonization. Another 16 noninoculated seedlings were treated with water and served as controls. The containers of inoculated and noninoculated seedlings were arranged separately in divided plastic houses. The seedlings were grown in the plastic houses at 26±4°C in the day and 18±5°C at night, with 60 to 80% relative humidity, and 1000 ± 200 µmoles photon m^{-2} s^{-1} PPFD (photosynthetic photon flux density) in the day. After 8 months of transplanting, the seedlings were sampled for

measurements of growth, root system morphology and pullout resistance.

Growth and root system morphology

Eight inoculated and eight noninoculated seedlings were randomly selected, respectively. The plant height and root collar diameter were measured with height ruler and digital caliper. The plant root systems were carefully exposed by complete excavation method (Böhm, 1979). Hand excavation with small spade was carried out to prevent root damage. The rooting depth, root numbers and length were measured. The root systems were cut off from the stems with pruning shears. For each root system, root morphological traits, that is, taproot length, total root length, external root surface root area and root tip number were measured with WinRHIZO*Pro* Image Analysis System (Regent Instruments, Quebec, Canada) (Bouma et al., 2000). WinRHIZO*Pro* is an image analysis system for automatic root measurement in morphology (that is, root length, root surface area, root tip numbers, etc.). Total root volume was measured with water displacement method (Pang et al., 2011). Roots and stems were then dried in an oven at 75°C till a constant weight. Root functional traits, that is, root density (RD), root length density (RLD), root tissue density (RTD), specific root length (SRL), and root to shoot ratio (R/S), were calculated by using the following formulas (Burylo et al., 2009, 2012; Strokes et al., 2009; Gould et al., 2016):

$$RD\ (kg\ m^{-3}) = \frac{}{} \quad (1)$$

Where, RD is the root density, DW_R is the total root dry weight, and V is the unit volume of root-permeated soil.

$$RLD\ (km\ m^{-3}) = \frac{}{} \quad (2)$$

Where, RLD is the root length density, L_R is the total root length measured using WinRHIZO, and V is the unit volume of root-permeated soil.

$$RTD\ (g\ cm^{-3}) = \frac{}{} \quad (3)$$

Where, RTD is the root tissue density, DW_R is the total root dry weight, and V is the total root system volume measured using WinRHIZO.

$$\textbf{SRL (m g}^{-1}\textbf{)} = \frac{}{} \quad (4)$$

Where, SRL is the specific root length, L_R is the total root length measured using WinRHIZO, and DW_R is the total root dry weight.

$$\textbf{R/S} = \frac{}{} \quad (5)$$

Where, R/S is the root to shoot ratio, DW_R is the total root dry weight, and DW_S is the total shoot dry weight.

Pullout test

After 8 months of transplanting, the rest of the eight seedlings each of inoculated and noninoculated seedlings were used for pullout test. The soil material was classified as sandy loam (containing 67.5% sand, 9.5% clay and 23% silt; specific gravity 2.63; bulk density 1.41 g/cm^3, porosity 45%). The average soil dry weight was 12.4 kN/m^3 and the average soil moisture content was 25%. Before pullout test, plant height and root collar diameter were measured. The plant stem was cut off at 10 cm above the root collar, and the bark was girdled and removed to prevent slippage of the special pulling fixture (Figure 3). The *in situ* pullout instrument (U-Soft USPA-003, U-Soft Tech, Taiwan) was used in pullout test. The load cell (Kyowa 5T, LUK-5TBS, Kyowa, Tokyo, Japan) was connected with the loading recorder and constant pullout control unit (rate adjustable from 2 to 4 mm/min), and mounted on a portable triangular steel frame. The data of pullout resistance and displacement were acquired through a portable computer system. Then, the instrument was connected to the pulling fixture. A constant vertical pulling speed of 2 mm/min was then applied automatically until the resisting force dropped sharply.

Data analysis

Variations in growth and morphological traits data between inoculated and noninoculated seedlings were analyzed using SPSS 22.0 for Windows (SPSS, Chicago, IL., USA) with t-test and one-way analysis of variance (ANOVA) Scheffé's method. The relationships between pullout resistance and plant traits were analyzed using Microsoft Excel regression analysis (version from Office 2013).

RESULTS

Rhizobial strain isolation, sequencing and phylogenetic analysis

The rhizobial strain isolated and purified was designated as AC1. Molecular analysis revealed that the 16S rRNA gene sequence of the isolated strain AC1 has 100% similarity to that of *Bradyrhizobium elkanii* (Huang et al., 2012) and was thus classified into the same group by maximum-parsimony and neighbor joining methods (Figures 1 and 4). Thus, the local strain AC1 was identified as *B. elkanii*. Inoculation test with AC1 revealed that *B. elkanii* effectively induces nodule formation in the roots of *A. confusa* (Figure 5).

Seedling growth

Statistical analysis revealed that inoculation significantly influenced most morphological parameters (Table 1). On average, inoculated seedlings had significantly larger height (40%), tap root length (100%), root biomass (130%), and shoot biomass (140%) than the noninoculated controls, but inoculation did not significantly affect root-collar diameter of seedlings (Table 1).

Root system morphology

The inoculated *A. confusa* seedlings developed bigger and deeper root systems than the noninoculated controls (Figure 6). The pattern of root growth in the inoculated seedlings showed that the maximum root developed to a depth of 70 cm, with more than 80% of the root matrix observed in the top 30 cm, and the lateral roots grew

Score	Expect	Identities	Gaps	Strand
1240 bits(671)	0.0	671/671(100%)	0/671(0%)	Plus/Plus

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Query  1    CATAGCAATATGTCAGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CATAGCAATATGTCAGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCG  60

Query  61   GAACAACTGAGGGAAACTTCAGCTAATACCGGATAAGCCCTTACGGGGAAAGATTTATCG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GAACAACTGAGGGAAACTTCAGCTAATACCGGATAAGCCCTTACGGGGAAAGATTTATCG  120

Query  121  CCGAAAGATCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGAC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CCGAAAGATCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGAC  180

Query  181  GATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACT  240
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Sbjct  181  GATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACT  240

Query  241  CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGC  300

Query  301  CGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTGTGCGGGAAGATAATGACGG  360
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Sbjct  301  CGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTGTGCGGGAAGATAATGACGG  360

Query  361  TACCGCAAGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGC  420
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Sbjct  361  TACCGCAAGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGC  420

Query  421  TAGCGTTGCTCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGGTCTTTAAGTCAGGGGT  480
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Sbjct  421  TAGCGTTGCTCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGGTCTTTAAGTCAGGGGT  480

Query  481  GAAATCCTGGAGCTCAACTCCAGAACTGCCTTTGATACTGAAGATCTTGAGTTCGGGAGA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GAAATCCTGGAGCTCAACTCCAGAACTGCCTTTGATACTGAAGATCTTGAGTTCGGGAGA  540

Query  541  GGTGAGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCAGTGG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GGTGAGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCAGTGG  600

Query  601  CGAAGGCGGCTCACTGGCCCGATACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  CGAAGGCGGCTCACTGGCCCGATACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAG  660

Query  661  GATTAGATACC  671
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Sbjct  661  GATTAGATACC  671
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Figure 1. Partial sequence of strain AC1 16S ribosomal RNA gene (query), compared to that of *Bradyrhizobium elkanii* (subject).

horizontally and profusely (Figure 6a). In contrast, for the noninoculated seedlings, its maximum root extended only to a depth of 50 cm, with more than 90% of the root matrix confined to the top 20 cm, and its lateral roots extended sparsely (Figure 6b). The observed root system morphologies of both inoculated and noninoculated *A. confusa* seedlings were classified to the VH-type.

Analysis of morphological parameters by WinRHIZO showed that inoculation significantly affected every morphological parameter. On average, the inoculated seedlings had significantly larger total root length (150%), external root surface area (130%), root volume (70%), and root tip number (60%) than the noninoculated controls (Table 2).

Analysis of root functional traits showed that the root density (RD), root length density (RLD) and root to shoot biomass ratio (R/S) of *A. confusa* seedlings inoculated with *B. elkanii* AC1 were significantly higher than the control ones. On average, seedlings inoculated with *B. elkanii* had significantly higher RD (130%), RLD (60%) and SLR (60%) than the controls. However, inoculation did not affect RTD and R/S of seedlings (Table 3).

Pullout resistance

In total, eight replicated pullout tests were performed to investigate the pullout resistance of the inoculated and

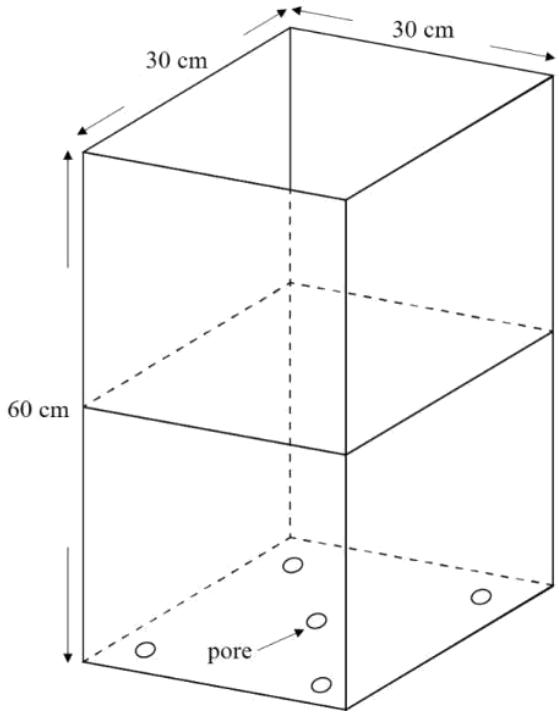

Figure 2. Plant culture box.

Figure 3. Special pulling fixture.

noninoculated *A. confusa* seedlings. The average maximum pullout resistance of the inoculated seedlings was 1.28 ± 0.11 kN, which was significantly higher than that of the noninoculated controls (0.58 ± 0.10 kN) (Table 4). The pullout resistance-displacement curves showed a steep slope up to the maximum force before the roots broke (Figure 7). Regression analysis revealed the linear positive relationship between pullout resistance and tree height (Figure 8), taproot length (Figure 9) and root biomass (Figure 10), that is, the pullout resistance increased with increasing tree height, taproot length and root biomass, with the root biomass showing the strongest relation. Meanwhile, pullout resistance also had a linear positive relationship with stem basal diameter and shoot biomass (Table 5).

DISCUSSION

In this study, the native rhizobial strain associated with *A. confusa* was isolated and identified as *B. elkanii* by 16S rRNA gene similarity analysis. Inoculation test demonstrated that *B. elkanii* effectively induces nodule formation in the roots of *A. confusa*. Many studies classified the rhizobia associated with *Acacia* spp. into three groups, that is, *Rhizobium-Sinorhizobium-Mesorhizobium* (that is, *A. Senegal*, *A. raddina* and *A. cyanophylla*, respectively) (De Lajudie et al., 1994, 1998; Khbaya et al., 1998), *Bradyrhizobium* (that is, *A. albida*, *A. mangium* and *A. auriculiformis*, respectively) (Galiana et al., 1990; Dupuy et al., 1994; Ferro et al., 2000), and two types of rhizobia (that is, *A. Seyal*) (Dreyfus and Dommergues, 1981). Diouf et al. (2015) studied the genetic and genomic diversity of *Acacia* mesorhizobia symbionts in Senegal, and discovered three new species of *Mesorhizobium*. Wang et al. (2008) indicated that rhizobia isolated from *A. mangium* in Fujian and Guangdong, China belongs to *Mesorhizobium*, whereas the strain isolated from *A. confusa* in Guangdong belonged to *Bradyrhizobium*. Huang et al. (2012) isolated a novel strain D5 from China, which is a new type of nitrogen-fixing bacterium from the nodules of *A. confusa*. In this study, the strain isolated from the local *A. confusa* was identified as *B. elkanii*, and we demonstrated in this study for the first time that it can effectively nodulate the roots of *A. confusa* seedlings.

Our functional study demonstrated that inoculation of *A. confusa* seedlings with the indigenous *B. elkanii* significantly enhanced seedling growth as compared to the non-inoculated controls. In the past, several studies have shown that inoculation of *Acacia* with *Bradyrhizobium* spp. significantly enhance growth of *Acacia* seedlings (Galiana et al., 1990, 1994; Dumroese et al., 2009; Pereyra et al., 2015). Martin-Laurent et al. (1999) showed that inoculation with the indigenous Malaysian strains *Bradyrhizobium* spp. significantly enhances seedling survival, growth and leaf chlorophyll content of *A. mangium* in the field. They also suggested

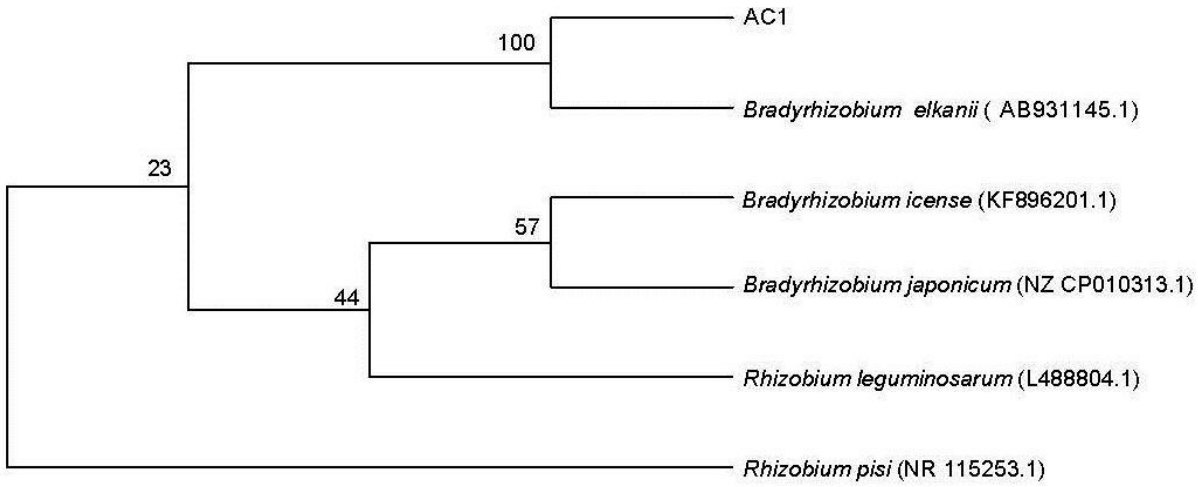

Figure 4. Phylogenetic relationship constructed by maximum-Parsimony (MP) methods based on 16S rRNA sequence of strain AC1. *Rhizobium pisi* was rooted as an out group.

Figure 5. Nodules formed on the roots of *A. confusa* inoculated with *B. elkanii* (bar = 1 cm).

Table 1. Plant morphological parameters of *A. confusa* seedlings inoculated and noninoculated with *B. elkanii* after 8 months of transplanting in the plastic house.

Treatment	Height (cm)	Root-collar diameter (mm)	Tap root length (cm)	Root biomass (g)	Shoot biomass (g)
In	251.7±5.5^{a}	17.9±0.3^{a}	89.0±13.0^{a}	64.0±16.7^{a}	152.3±18.2^{a}
C	179.0±4.0^{b}	16.2±2.4^{a}	44.3±5.1^{b}	28.0±5.5^{b}	64.3±26.1^{b}

In, inoculated; C, noninoculated control. All values are the mean ± standard error of eight replicates. Values in the same column with different superscript letters significantly differ at 5% significant level.

that indigenous *Bradyrhizobium* strains may be more efficient in competition with other strains. Several other studies also revealed the beneficial effects of *Bradyrhizobium* symbiosis on plant growth and biomass (Glick, 1995; Usharani et al., 2014). Consistent with these observations, our results also shows the positive effect of

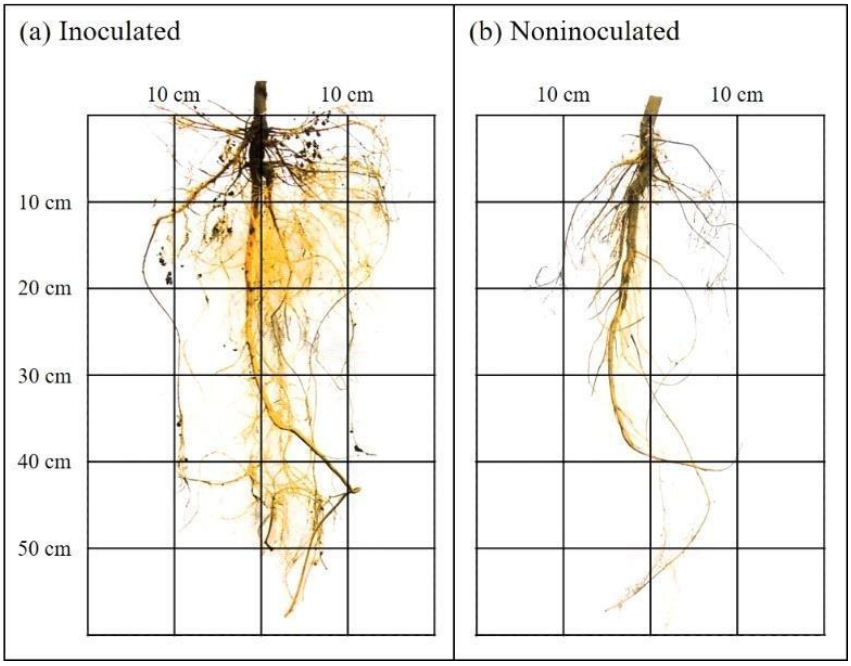

Figure 6. Root system morphologies of *A. confusa* seedlings after 8 months of transplanting in the plastic house. **(a)** Inoculated. **(b)** Noninoculated.

Table 2. Root morphological traits for *A. confusa* seedlings inoculated and noninoculated with *B. elkanii* after 8 months of transplanting in the plastic house.

Treatment	Total root length (cm)	External root surface area (cm^2)	Root volume (cm^3)	Root tip number
In	2687.5±598.2^a	1345.0±210.2^a	275.0±75.7^a	5356.0±764.3 ^{a}
C	1066.0±196.9^b	587.5±132.6 ^{b}	163.3±30.6^b	3324.2±343.2 ^{b}

In, inoculated; C, non-inoculated control. All values are the mean ± standard error of eight replicates. Values in the same column with different superscript letters significantly differ at 5% significant level.

Table 3. Root functional traits of *A. confusa* seedlings inoculated and non-inoculated with *B. elkanii* after 8 months of transplanting in the plastic house.

Treatment	RD (kg m^{-3})	RLD (km m^{-3})	RTD (g cm^{-3})	SRL (m g^{-1})	R/S
In	1.19±0.31^{a*}	0.29±0.11^a	0.16±0.07^a	0.45±0.26^a	0.41±0.09^a
C	0.52±0.10^b	0.18±0.05^b	0.26±0.21^a	0.28±0.10^b	0.43±0.07^a

RD, Root density; RLD, root length density; RTD, root tissue density; SRL, specific root length; R/S, root to shoot ratio; In, inoculated; C, noninoculated control. All values are the mean ± standard error of 8 replicates. Values in the same column with different superscript letters significantly differ at 5% significant level.

Table 4. Pullout resistances of inoculated and noninoculated *A. confusa* seedlings after 8 months of transplanting in the plastic house.

Treatment	Pullout resistance (kN)
In	1.28 ± 0.11 ^{a}
C	0.58 ± 0.10 ^{b}

In, inoculated; C, noninoculated control. All values are the mean ± standard error of 8 replicates. Values in the same column with different superscript letters significantly differ at 5% significant level.

B. elkaniii on growth of *A. confusa* seedlings.

The root system morphologies of the inoculated and non-inoculated *A. confusa* seedlings in this study resembled the VH-type root system, according to previous classification (Yen, 1987; Styczen and Morgan, 1995). The inoculated seedlings developed longer taproot and more profused lateral roots than the noninoculated controls. WinRHIZO analysis revealed that inoculation with *B. elkanii* significantly influence root morphological traits of *A. confusa* seedlings. Inoculation significantly

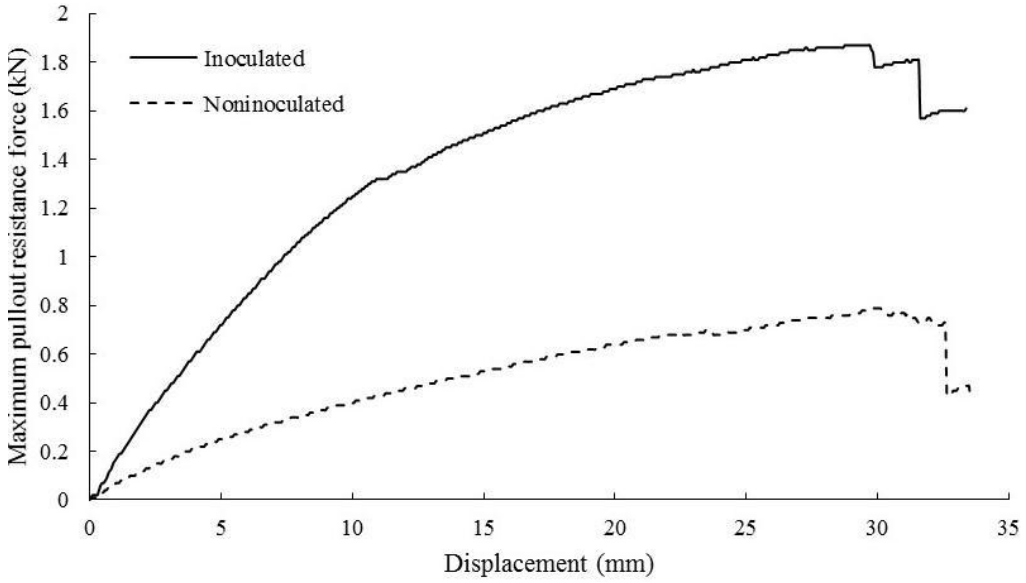

Figure 7. Pullout resistance force-displacement curves for *Acacia confusa* inoculated (-) and noninoculated (-)-with *B. elkanii*.

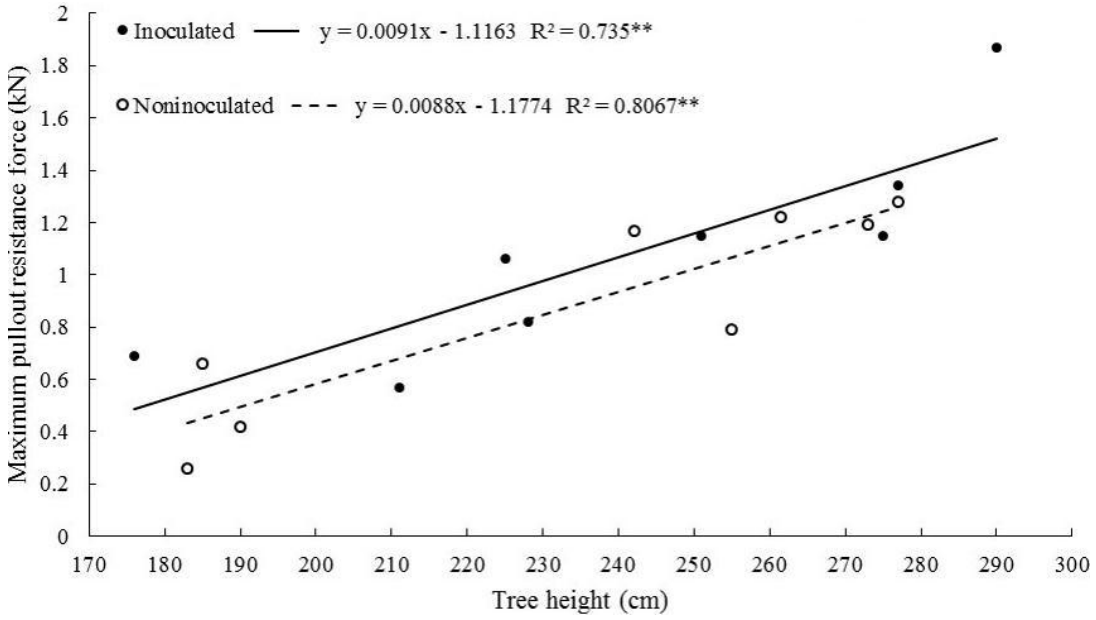

Figure 8. The relationship between the maximum pullout resistance force and tree height in the investigated *A. confusa* inoculated(●-)-and noninoculated (○)-with *Bradyrhizobium elkanii*. N = 8.

enhanced the total root length, external root surface area, root volume, and root tip number, RD, RLD and SRL, as compared to the controls; whereas inoculation had no significant effect on RTD and R/S. Stokes et al. (2009) indicated that seedlings with high RLD and SRL are desirable and useful to landslide restoration.

Our data clearly shows that inoculation with *B. elkanii* significantly increases the maximum pullout resistance of *A. confusa* as compared to the noninoculated controls, suggesting a superior root anchorage capability by the inoculated seedlings. Linear positive relationships were observed between pullout resistance and tree height, taproot length and root biomass, root collar diameter and shoot biomass. This study demonstrates that the inoculated *A. confusa* seedlings have developed longer taproot and more extensive number of lateral roots than

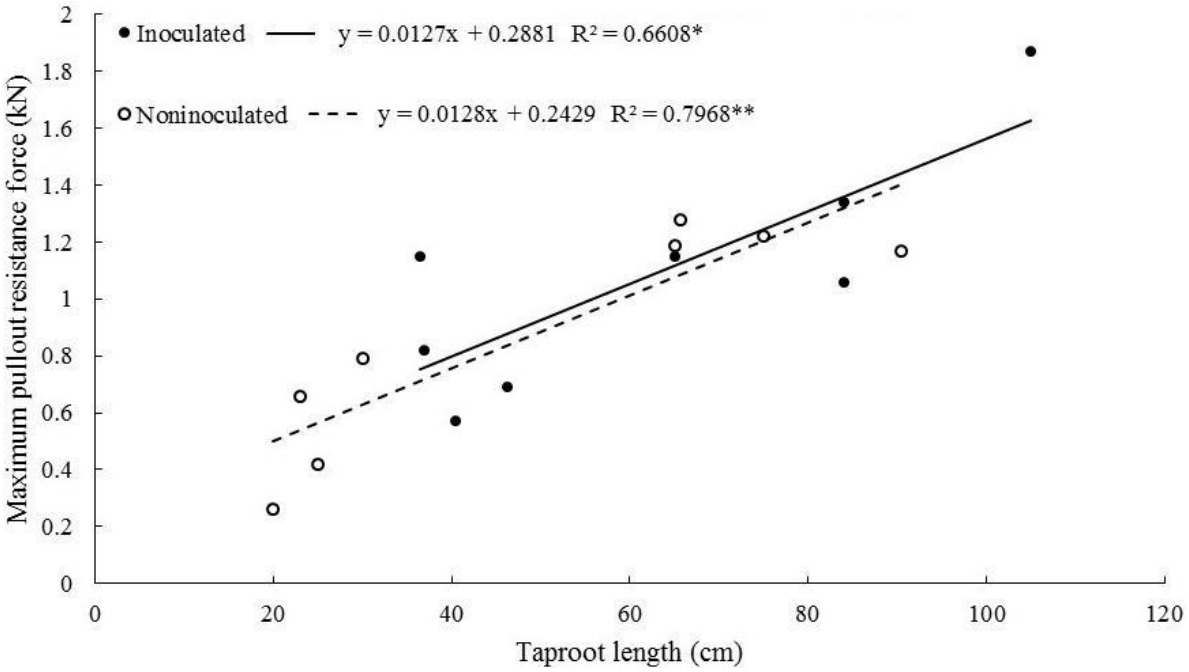

Figure 9. The relationship between the maximum pullout resistance force and taproot length in the investigated *Acacia confusa* seedlings inoculated (●) and non-inoculated (○) with *B. elkanii*. N = 8.

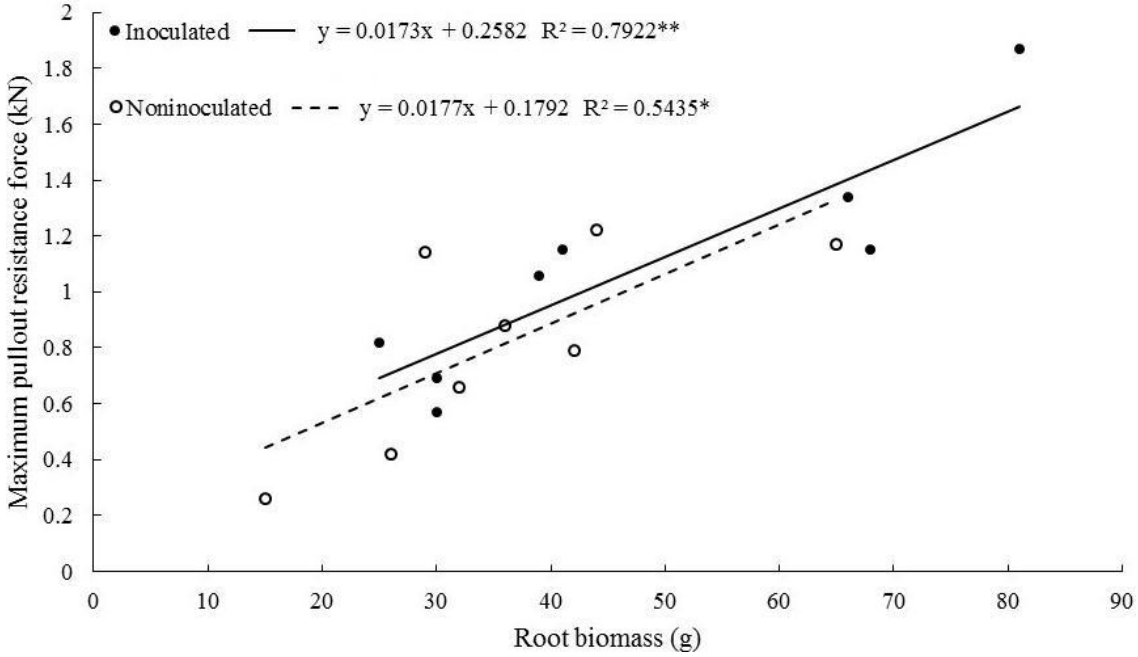

Figure 10. The relationship between the maximum pullout resistance force and root biomass in the *A. confusa* seedlings inoculated (●) and noninoculated (○) with *B. elkanii*. N = 8.

the noninoculated controls. Thus, inoculation with *B. elkanii* significantly increased the numbers of lateral roots, which promote plant anchorage and pullout resistance to vertical uprooting forces. Also, inoculated *A. confusa* seedlings developed VH-type root system, which is the most resistant to uprooting (Dupuy et al., 2005;

Table 5. Relation between morphological traits and pullout resistance of *A. confusa* inoculated and noninoculated with *B. elkanii* after 8 months of transplanting in the plastic house.

Morphological traits	Treatment	Mean±SE	Regression equation	R^2	*p*
H	In	247.0±26.9[a]	y=0.009x-1.116	0.74	0.007
	C	212.5±28.8[b]	y=0.009x-1.177	0.81	0.002
D_r	In	18.53±1.48[a]	y=0.114x-1.350	0.75	0.027
	C	17.92±1.64[a]	y=0.105x-0.735	0.68	0.045
R_b	In	41.4±16.6[a]	y=0.017x+0.258	0.79	0.003
	C	33.8±7.6[a]	y=0.018x+0.179	0.54	0.037
S_b	In	128.8±36.6[a]	y=0.008x+0.255	0.74	0.006
	C	75.4±19.2[b]	y=0.008x+0.261	0.68	0.011
L_{tr}	In	79.1±22.1[a]	y=0.013x+0.288	0.66	0.014
	C	51.7±13.3[b]	y=0.013x+0.243	0.80	0.003

H, Tree height; D_r, root collar diameter; R_b, root biomass; S_b, shoot biomass; L_{tr}, taproot length; In, inoculated; C, noninoculated control; y, pullout resistance. All values are the mean ± standard error of 8 replicates. Values in the same column with different superscript letters significantly differ at 5% significant level.

Yen, 1987). Ennos et al. (1993) indicated that taproot acted like a stake guyed by lateral roots to resist vertical uprooting. Stokes et al. (2009) indicated that increased RLD augments the pullout resistance of seedlings.

Conclusions

In this study, a local rhizobial strain isolated from root nodules of *A. confusa* was identified as *B. elkanii*. Inoculation test revealed that the nitrogen-fixing *B. elkanii* can effectively nodulate the roots of *A. confusa*, and enhance the growth of its seedlings. Root excavation study showed that the root system morphologies of both inoculated and noninoculated *A. confusa* belonged to the VH-type root system, but the inoculated seedlings developed deeper taproot and more lateral roots than the non-inoculated controls. On average, inoculated seedlings had significantly larger total root length, external root surface area, root volume, and root tip number than the non-inoculated ones. Root functional traits such as RD, RLD and SRL of the inoculated seedlings were significantly higher than that of the controls. The average maximum pullout resistance of the inoculated *A. confusa* was more than twice higher than that of the non-inoculated ones. Regression analysis showed the linear positive relationship between pullout resistance and tree height, taproot length, root biomass, root collar diameter and shoot biomass. Taken together, these results clearly demonstrate that the local strain of the nitrogen fixing *B. elkanii* can significantly promote growth, root morphology and pullout resistance of *A. confusa* after eight months of transplanting. These findings are important in the application of *B. elkanii* in *A. confusa* agroforestry and plantation forestry as well as soil conservation engineering. This is the first report to show a positive effect of inoculation with *B. elkanii* on the growth, root development and root anchorage capability of *A. confusa*. Furthermore, future studies on the symbiotic compatibility and nitrogen-fixing ability between *B. elkanii* and other acacia species, that is, *A. auriculiformis*, *A. farnesiana and A. mangium* are needed for tree nursery applications. Also, researches on the influences of abiotic factors, that is, soil pH, fertility, and drought on the symbiotic association are useful in reforestation practices.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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