

Promoting Role of *Bacillus Subtilis* BS87 on the Growth and Content of Some Natural Products in the Medicinal Plants *Anoectochilus Roxburghii* and *A. Formosanus*

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Abstract *Anoectochilus* genus is an epiphytic Orchid used as a traditional Chinese folk medicine for the treatment of many diseases. Plant growth-promoting rhizobacteria (PGPR) such as *Bacillus subtilis* play an important role in promoting medicinal orchids growth. This study aimed to investigate the promoting role of *B. subtilis* BS87 on the biomass and contents of some natural products in *A. roxburghii* and *A. formosanus* such as flavonoids, steroids and essential oils, in *A. roxburghii* and *A. formosanus* treated for 90 days with *B. subtilis* BS87, the flavonoid contents reached 1.208% and 1.424%, respectively, whereas the steroid content in both species was closely values approximately. The essential oil content was 0.11% was 0.21%, respectively, which was significantly different to levels in control plants in pot culture. After 90 days of symbiotic cultivation, increased shoot height, fresh weight and leaf number were observed in treated *A. roxburghii* and *A. formosanus* plants compared with control plants. In addition, qRT-PCR showed that the expression levels of seven plant growth-related genes, namely AR017, AR019, AR020, AR023, AR037, AR044, and AR047 were increased in both *A. roxburghii* and *A. formosanus* plants treated with *B. subtilis* BS87 90 days post-inoculation compared with control plants.

Keywords *Anoectochilus formosanus*, *Anoectochilus roxburghii*, *Bacillus subtilis* BS87, Essential oils, Flavonoids, Steroids, Natural products, *Anoectochilus*

1. Introduction

Anoectochilus is a genus of about 40 orchids (family Orchidaceae) in the tropical zone areas of Asia and Australia. The genus includes 20 species; two varieties have been found in southwestern and southern China [1]. The Asian plants *A. formosanus* and *A. roxburghii* are distributed throughout China, Taiwan, and Japan, which used for the treatment of many diseases including fever, pleurodynia, lung, snake bite, liver disease, hypertension, and malnourishment in children [2-4]. Moreover, *A. formosanus* is used to treat many diseases such as chest and abdominal pain, nephritis, impotence, spleen as an anti-inflammatory agent [5, 6], and is used to possess antioxidant activity and in the treatment of cancer [7, 8], liver disease [9], hepato toxicity [10], and diabetes [11]. It also increase endurance capacity [12]. The highly potent medicinal activity of

A. formosanus and *A. roxburghii* are due to their secondary metabolites, such as kinsenosides, flavonoids, steroids, and essential oils; the first kinsenosides were isolated from *A. koshunensis* [13]. Another study assessed the essential oil from *A. roxburghii* and its effect on inhibiting hyperplasia of a human cell lung cancer cell line [14]. There are many organisms that are vital constituents of soil and affect different biotic activities of the soil ecosystem in terms of nutrient turnover and the potential for crop production [15, 16]. Bacteria of diverse genera have been identified as plant growth promoting rhizobacteria (PGPR), such as *Bacillus* sp. The effect of PGPR on plant growth directly or indirectly leads to the promotion of plant growth, either by supplying the plant with a compound that is synthesized by the bacterium (for example plant hormones and plant growth regulators), facilitating the absorption of certain nutrients from the environment, protecting plants from pathogens, improving soil structure, bio remediating contaminated soils by sequestering toxic heavy metal species, and meeting xenobiotic components and the indirectly way as induced resistance in plants [17-19]. In earlier studies, growth genes have been shown to be involved in the growth of various

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plants after treatment with *Bacillus* sp.; *B. subtilis* OKB105 was found to affect the transcription of 176 genes involved in metabolism, the transport of nutrients, stress responses, chemotaxis, sporulation, teichuronic acid biosynthesis, and motility in rice seedlings [20]. Another study showed that rice proteins involved in plant growth were induced after exposure to *B. cereus* NMSL88 [21]. Several genes of *A. roxburghii* are differentially expressed during symbiosis with the mycorrhizal fungus *Epulorhiza* sp. (AR-18) compared with non-treated plants, where seedlings treated with the fungus for 35 days showed an average growth increase of 10.3% compared with non-treated seedlings [22]. The present study aimed to identify the optimum tissue culture medium and identify the plant growth-promoting activity of *B. subtilis* BS87 on *A. roxburghii* and *A. formosanus*. We investigated the promoting role of *B. subtilis* BS87 on the biomass and flavonoid, steroid and essential oil contents of *A. roxburghii* and *A. formosanus* in greenhouse experiments. These findings will help elucidate the effect of *B. subtilis* BS87 on the production of secondary metabolites in orchid plants.

2. Results

2.1. Effect of *Bacillus Subtilis* BS87 Bacteria on *A. Roxburghii* and *A. Formosanus* Vegetative Growth

All *A. roxburghii* and *A. formosanus* plants inoculated or control were harvested three months after cultivation to determine the influence of *Bacillus subtilis* BS87 on the growth of *A. roxburghii* and *A. formosanus* plants. The results show that *Bacillus subtilis* BS87 significantly affected the growth of micropropagated *A. roxburghii* and *A. formosanus* plants *in vitro* plants in pots, as the BS87 plants appeared to be more vigorous than the controls plants (Figure 1). The shoot height (cm), fresh weigh (g) and leaf number of BS87-colonized *A. roxburghii* plants were 11.25, 20.00 and 18.33, respectively; these values were 9.16, 17.94 and 8.00 in control plants (Table 1). The shoot height (cm), fresh weigh (g) and leaf number of BS87-colonized *A. formosanus* plants were 12.86, 24.10 and 35.33, respectively; these values were 8.34, 18.84 and 12.67 in control plants (Table 1).

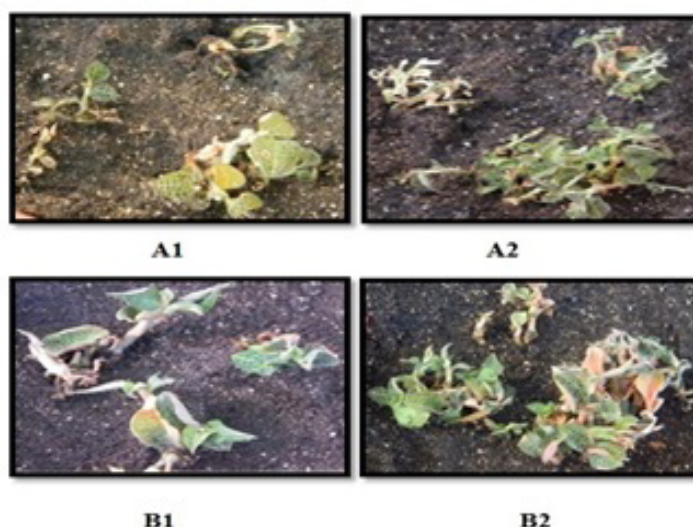


Figure 1. Effect of *Bacillus subtilis* BS87 on *Anoectochilus* growth after three months. A- *A. roxburghii* (A1) without *B. subtilis* BS87.(A2) with BS87 B-*A. formosanus* (B1) without *B. subtilis* BS87.(B2) with BBS87

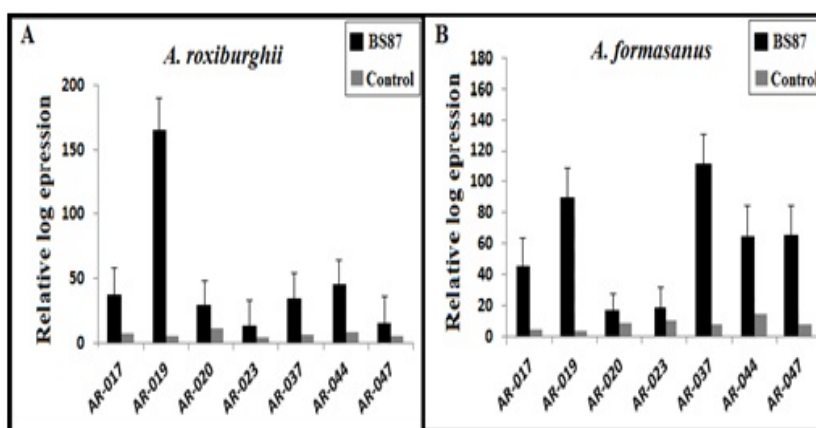


Figure 2. Expression of growth-related genes after treatment with *B. subtilis* BS87 or with water as a control treatment in 90-day-old *Anoectochilus roxburghii* and *A. formosanus* plants

Table 1. Effect of *Bacillus subtilis* BS87 on *A. roxburghii* and *A. formosanus*

Treatment	Shoot height (cm)	Fresh weigh (g)	Leaf No.
<i>A.roxburghii</i>	9.16 ±0.02	17.94±0.04	8.00±0.05
<i>A.roxburghii</i> + BS87	11.25±0.15	20.00±0.05	18.33±0.13
<i>A.formosanus</i>	8.34±0.10	18.84±0.12	12.67±0.25
<i>A.formosanus</i> +BS87	12.86±0.07	24.10±0.05	35.33±0.32

2.2. *Bacillus Subtilis* BS87-Induced Gene Expression

Increased growth in plant is associated with the expression of growth marker genes. To determine whether BS87 acts as an inducer of growth in *A. roxburghii* and *A. formosanus* under greenhouse conditions, we used the genes *AR-017*, *AR-019*, *AR-020*, *AR-023*, *AR-037*, *AR-044*, and *AR-047* as markers for the growth plants in this experiment. The expression of these selected genes was assessed 90 days after inoculation using quantitative real time-polymerase chain reaction (qRT-PCR) (Figure 2). The expression of these genes increased in *A. roxburghii* (Figure 3 A) and *A. formosanus* plants (Figure 2 B) after 90 days of treatment with 10 ml of *B. subtilis* BS87 compared with control plants. The expression of *AR-019* increased with a peak in *A. roxburghii* plants (Figure 2, A) and the expression of *AR-037* increased with a peak in *A. formosanus* plants (Figure 2, B) treated with *B. subtilis* BS87 (Figure 2, A and B). The expression of *AR-020*, *AR-037*, *AR-044*, and *AR-047* were increased in *A. formosanus* compared with *A. roxburghii* plants at 90 days post inoculation with *B. subtilis* BS87, whereas the expression of *AR-017* and *AR-044* were increased in *A. roxburghii* compared with *A. formosanus* plants (Figure 2, A and B).

Total RNA was extracted and first-strand cDNA was

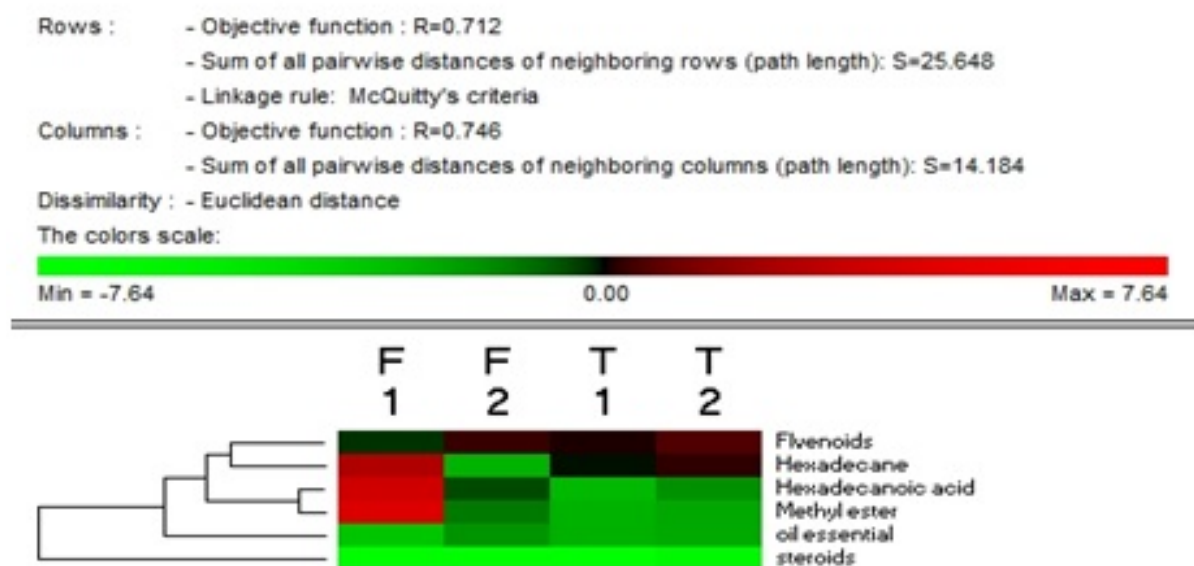
synthesized for expression analysis. Expression of the genes *AR-017*, *AR-019*, *AR-020*, *AR-023*, *AR-037*, *AR-044* and *AR-047* was monitored by qRT-PCR. Each experiment was repeated at least three times.

2.3. Effects of *Bacillus subtilis* BS87 Bacteria on Flavonoids, Steroids and Essential Oil Compounds in *A. Roxburghii* and *A. Formosanus*

In this experiment, we studied the content of flavonoids, steroids and essential oil compounds in *A. roxburghii* and *A. formosanus* 90 days post-inoculation with BS87 bacteria compared with control. The results showed that the flavonoid content reached 0.850 and 1.208% in *A. roxburghii* control plants and treated with *Bacillus subtilis* BS87, respectively, while levels reached 1.050 and 1.424%, respectively, in *A. formosanus*. The contents of steroid compounds differed significantly between control plants and treated plants, i.e.0.007 and 0.008%, respectively, in *A.roxburghii* and 0.005 and 0.013%, respectively, in *A. formosanus*. The content of essential oil compounds differed significantly between control plants and treated plants with *Bacillus subtilise* 87, i.e.0.11 and 0.30%, respectively, in *A. roxburghii* and 0.18 and 0.21%, respectively, in *A. formosanus* (figure 3). And there is a common material in the installation each oil essential for *A.roxburghii* and *A. formonosanus* with and without treated *Bacillus subtilis* which were Hexadecane, Hexadecanoic acid and Methyl ester (Figure 3).

F1, T1- the contents of products for *Anoectochilus roxburghii* and *Anoectochilus formosanus* respectively in greenhouse experiments after 90 days control plants.

F2, T2-the contents of products for *Anoectochilus roxburghii* and *Anoectochilus formosanus* respectively in greenhouse experiments after 90 days treated with bacteria.

**Figure 3.** Effects of *B. subtilis* BS87 bacteria on flavonoids, steroids and essential oil compounds in *A. roxburghii* and *A. formosanus*

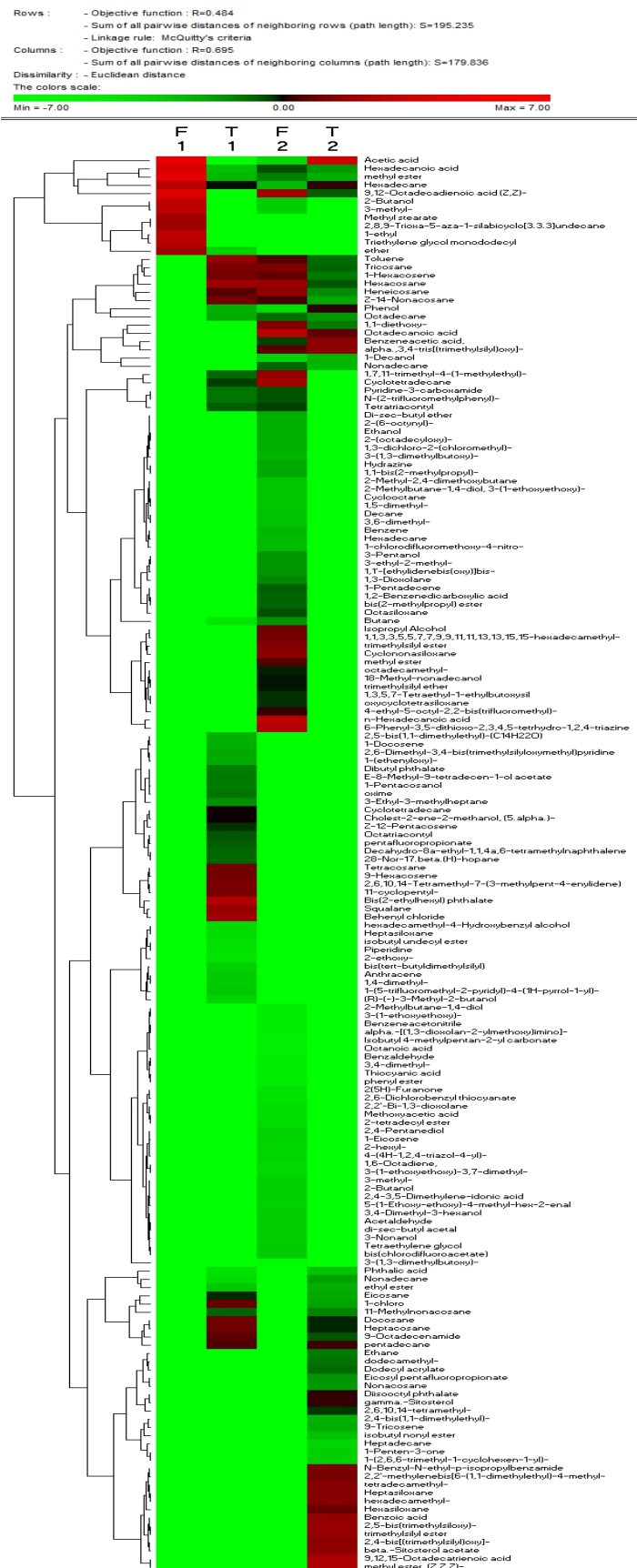


Figure 4. Essential oils contents for *Anoectochilus roxburghii* and *Anoectochilus formosanus* in greenhouse experiments after 90 days, treated with and control plants

The essential oil composed from different chemical components. The major components of the essential oils extracted from *A. roxburghii* with bacteria and without bacteria were fatty acids such as hexadeconic acid, weak acids such as acetic acid, alkanes such as hexadecane, esters such as methyl ester, and alcohols such as 2-butanol. The major components of the essential oils extracted from *A. formosanus* with bacteria and without bacteria were alkanes such as hexadecane, nonadecane, octadecane, 11-methylnonacosane, heneicosane, docosane, tricosane, pentadecane, hexacosane, z-14-nonacosane, and heptacosane, esters such as methyl ester and ethyl ester, alcohols such as 3-methyl-2-butanol. Moreover, the extract contained toluene (a liquid aromatic hydrocarbon C₇H₈ that resembles benzene but is less, phenol (an extremely poisonous compound), phthalic acid (anisomeric acid), eicosane (an isomeric hydrocarbon), and alkenes such as 1-hexasene (Figure 4).

F1, T1- Essential oils contents for *Anoectochilus roxburghii* and *Anoectochilus formosanus* respectively in greenhouse experiments after 90 days control plants.

F2, T2- Essential oils contents for *Anoectochilus roxburghii* and *Anoectochilus formosanus* respectively in greenhouse experiments after 90 days treated with bacteria.

3. Discussion

This genus is included *A. formosanus*, *A. roxburghii*, and *A. koshunensis*, which are generally used in the treatment of some diseases [1, 23]. *Anoectochilus* species have a unique mycorrhizal relationship and complex interactions with fungal groups such as Rhizoctonia, Agaricales, and Ascomycetes.

These interactions have been related to host plant growth, yield, fitness, and stress responses [24-27]. Has been observed in *A. formosanus* Hayata. *Bacillus subtilis* BS87 has not been tested in practice on medicinal plants. However, as a PGPR, it can lead to increased growth and induced resistance in plants. Therefore, we analyzed the effect of this bacteria in *A. roxburghii* and *A. formosanus* under greenhouse conditions. We observed that *B. subtilis* BS87 induced the expression of the plant growth-related genes *AR-017*, *AR-019*, *AR-020*, *AR-023*, *AR-037*, *AR-044*, and *AR-047* compared with control plants. It was also noted that the bacteria stimulated plant growth, indicated by the wet weight and plant height as compared with non-treatment plants, thus demonstrating the broad range of activity of *Bacillus subtilis* BS87.

These results are consistent with several studies that showed the ability of *Bacillus subtilis* to stimulate the growth of different plants. *Bacillus subtilis* has been widely studied for the improvement of plant growth under both natural and controlled conditions [28], and strains of *Bacillus subtilis* have been shown to promote tomato and corn plant growth and induce *At4g36110*, *At2g46370*, and *At1g69490* gene expression in *Arabidopsis* [29]. Therefore, we

hypothesized that the increase in *A. roxburghii* and *A. formosanus* biomass may have been due to the influence of BS87 on photosynthesis and enzyme activity in the host plants. Here, we assessed the expression of candidate growth genes in *A. roxburghii* and *A. formosanus* leaves collected from plants treated or not with *Bacillus subtilis* BS87 by qRT-PCR. We used GAPDH primer as the housekeeping gene to ensure the validity of expression analysis in this experiment.

We found a significant increase in the expression of all growth genes in *A. roxburghii* and *A. formosanus* treated with *Bacillus subtilis* BS87 compared with control plants. Such increases in gene expression occurred in response to BS87, thereby suggesting that host plants quickly respond to elicitors of growth such as *Bacillus subtilis* BS87. In previous studies, five genes, i.e. *AR-DD017*, *AR-DD019*, *AR-DD037*, *AR-DD044*, and *AR-DD047*, including genes encoding a hypothetical protein and a uracil phosphor ribosyl transferase, were up-regulated in *A. roxburghii* (60 days old) treated with the mycorrhizal *Epulorhiza* sp. fungus (AR-18), assessed by differential display reverse transcription (DDRT)-PCR. Seedlings grown with *Epulorhiza* sp. AR-18 for 35 d showed an average growth increase of 10.3% compared with non-treated seedlings [22]. These results are consistent with our results regarding the expression of genes associated with the growth in *A. roxburghii* and *A. formosanus*. The results of the present study show that *A. roxburghii* and *A. formosanus* treated with *Bacillus subtilis* BS87 gave the best results in terms of increased contents of flavonoids, steroids and essential oil compounds compared to plants control after 90 days of inoculation. Recent research has demonstrated that the essential oils of *A. roxburghii* are biologically active and may function as antioxidants as well as anti-inflammatory and anti-tumor agents [30-32]. *Anoectochilus* was analyzed and two species in this genus were compared. The results showed that, using an HPLC rapid test of flavonoid aglycones, two kinds of flavonoid aglycones from *A. formosanus* could be detected simultaneously [3].

4. Materials and Methods

4.1. Plant and Bacterial Materials

A. roxburghii, *A. formosanus* and *B. subtilis* BS87 used in this study were stored in our research laboratory. *A. roxburghii* and *A. formosanus* explants were grown on improved MS medium [33], supplemented with 3% (w/v) sucrose, and 0.75% (w/v) agar, at pH 5.8. Explants were grown in flasks with a 12h light/12h dark photoperiod at (24±1)°C. *B. subtilis* BS87 was grown in conical flasks (300 ml) containing 150 ml of *B. subtilis* at 48 h on a rotary shaker (150 rpm) at (28± 2°C). The density of the suspension was set at 2×10⁷ colony forming units (cfu)/ml [34]. Pure cultures of bacteria were maintained on respective agar slants and stored at 4°C for further use.

4.2. Plant Inoculation

Experiments were designed under greenhouse conditions using the micropropagation of *A. roxburghii* and *A. formosanus*. After 90 days of micropropagation *in vitro*, aseptic plants were grown in a greenhouse in 8 cm × 8 cm × 9 cm plastic pots containing 1 kg of a peat moss/humus soil mixture at a ratio of 1:1 that had been inoculated with *B. subtilis* BS87 (2×10^7 cfu) at a dose of 10 ml/pot, then mixed with the upper soil surface in each pot. Other *A. roxburghii* and *A. formosanus* plants were planted separately in sterile soils not treated with bacteria as the control treatment. Plants were grown at 20-25°C with a 12 h/12 h light/dark cycle and 62.5% humidity. Irrigation was applied by drenching twice a week. Each group consisted of five pots with three to four *in vitro* micropropagated plants per pot.

Table 2. PCR primers used in the present study

Gene (s)	Accession number	Primer sequence (50–30)
AR-017	GR410423	Forward: AACCAAAATGTTTCGTCCCGTCT
		Reverse :GACGCCTACCGTTGCACTCG
AR-019	GR410424	Forward: AACCAAAATGTTTCGTCCCGTCT
		Reverse :GACGCCTACCGTTGCACTCG
AR-020	GR410425	Forward: AACCAAAATGTTTCGTCCCGTCT
		Reverse: GACGCCTACCGTTGCACTCG
AR-023	GR410426	Forward: AACCAAAATGTTTCGTCCCGTCT
		Reverse: GACGCCTACCGTTGCACTCG
AR-037	GR410427	Forward: AACCAAAATGTTTCGTCCCGTCT
		Reverse: GACGCCTACCGTTGCACTCG
AR-044	GR410428	Forward: AACCAAAATGTTTCGTCCCGTCT
		Reverse: GACGCCTACCGTTGCACTCG
AR-047	GR410429	Forward: AACCAAAATGTTTCGTCCCGTCT
		Reverse: GACGCCTACCGTTGCACTCG

4.3. Gene Expression Assay

Total RNA was isolated from 1 g of stem and leaf tissues from *A. roxburghii* and *A. formosanus* treated or control plants. *subtilis* at 90 days post inoculation. RNA was isolated using the RNeasy Plant Mini Kit (Qiagen 74904). After treatment with DNase I (New England M0303S) at 37°C for 30 min, 5 µl of RNA was used for first-strand cDNA synthesis using Super Script II reverse transcriptase, following the instructions of the manufacturer (In vitrogen 18064-014). All cDNA samples were stored at –20°C until used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. qRT-PCR was performed using an Applied Biosystems machine and Step One Software v2.3 (USA). The specific primers used for qRT-PCR are listed in

Table 1. The PCR reaction was performed using 1 µl of cDNA as the template in a total reaction volume of 20 µl in 0.5 ml PCR tube using iTaqTM SYBR[®] Green Super-mix with ROX (California, USA). Then, 0.3 µl of each primer and double-distilled H₂O were made up to a total volume of 8.4 µl and mixed in a 96-cell plate. The *GAPDH* primer was used as the control for the experiment (Table 2).

4.4. Extraction and Sample Preparation for *A. Roxburghii* and *A. Formosanus*

4.4.1. Measurement of Intracellular and Extracellular Total Flavonoid Contents for *A. roxburghii* and *A. formosanus*

After 90 days of growth in the greenhouse, 15 plants from each treatment group were harvested. Whole plant powder was prepared, and 0.1-1g was added to 4ml of cold 70% ethanol. The homogenate was centrifuged at 12,000 g for 5min at 4°C and the supernatant was used to measure the intracellular contents. The determination of the flavonoid content followed the methods of Jia and Eberhardt with modifications. The supernatant was diluted five-fold with 70% ethanol (1ml of supernatant in 4ml of 70% ethanol). Take rutin solution at the concentrations of 0.2,0.4,0.6,0.8 and 1.0 mg /L, and placed in a tube with 0.3ml of 5% NaNO₂ and 1ml of 70% ethanol; the contents were mixed and set aside for 6 min, then 0.3 ml of 4% Al(NO₃)₃ was added and the tube was set aside again for 6 min. Next, 2ml of 4% NaOH was added and measured at 510 nm after 10 min.

4.4.2. Measurement of Intracellular and Extracellular Total Steroid Contents for *A. roxburghii* and *A. formosanus*

To measure the steroid content, 0.2g of fresh plant materials was placed in a 100ml blue cap bottle, then 75 ml of n-hexane was added and magnetically stirred for 10 min. This was followed by 30min of ultrasonic extraction, then the supernatant was poured into two 50ml centrifuge tubes and allowed to stand. Next, 20ml of n-hexane was added to the residue and magnetically stirred for 5 min. The supernatant was transferred to a centrifuge tube, and centrifuged at 5000 rpm for 10 min. The supernatant was transferred to around bottom flask and concentrated to dryness at 40°C by rotary evaporation. The evaporation residue was dissolved in 25 ml of ethanol and 5ml KOH solution (500 g/L), refluxed for 30 min in a boiling water bath, then cooled to room temperature with running water immediately after the reaction. The saponification solution was transferred to a 200ml separator funnel (Teflon stopcock), then the round bottom flask was washed with 10ml of deionized water and combine the lotion into the separator funnel, then extracted three times with 50, 50 and 30 ml of petroleumether (the boiling point is 30-60°C). The organic phase was combined in a 200ml separator funnel, the 30ml of deionized water was added and the solution was mixed for 2 min. The lower aqueous layer was washed with de ionized water repeatedly until a neutral pH was achieved.

Then, 3-5g of an hydrous sodium sulfate was added to the extract and mixed for 2 min, then the extract was placed in a 100ml round bottom flask and concentrated to dryness by rotary evaporation. The unsaponifiable matter was then dissolved with glacial acetic acid and diluted to 5ml, and used as the sample solution. The absorbance was measured at 289 nm

4.4.3. Extraction of the Essential Oil

At least 10g of field culture plant material was washed with water to remove any debris, then cut into pieces. The sample was placed in a round flask and water was added to cover the sample. The bottles were placed in a reflux device, and the essential oil was distilled at 100°C (samples were heated with an electric jacket by setting the temperature to 200°C). Next, 5 ml of ether solute was placed in the receiving tube when steam was seen in the condensation tube. The ether and oil were collected in a new flask after 30 min. Another 5 ml of ether solute was placed in the receiving tube and collected after 20 min. This step was repeated many times in a period of 5 h. In the meantime, an NaCl solution was prepared (108g in 300 ml of distilled water). The volume of the water layer and the ether layer were equalized, and the NaCl solution was added to the collected solution (ether and oil) and shaken for 2min, then allowed to stand for several minutes until layer separation occurred. The upper layer was transferred to a new flask using a separator funnel. The chromatographic column was then prepared using sodium anhydrous sulfate. The collected solution was dried and the outflow liquid was collected in a 100 ml round distilling flask (if the volume was more than 50 ml, a 150 ml round distilling flask was used) and evaporated using a rotary evaporator. When the volume decreased to 25 ml, the solution was transferred to a previously weighed flask and evaporation was continued. The weight of the flask was measured again after evaporation.

5. Conclusions

It is necessary to find ways to protect, increase the growth, and enhance the yields of medicinal products from the *Anoectochilus* genus. We have found the application of *Bacillus subtilis* BS87 increased medicinal plant growth (*A. roxburghii* and *A. formosanus*), considering parameters such as shoot height, fresh weight and leaf number, and increased the yield of flavonoids, steroids and essential oil compounds. Then comparative between essential oil compounds after treated with Bacteria and with control plants. The results showed that the flavonoid content reached 0.850 and 1.208% in *A. roxburghii* control plants and treated with *Bacillus subtilis* BS87, respectively, while levels reached 1.050 and 1.424%, respectively, in *A. formosanus*. The contents of steroid compounds differed significantly between control plants and treated plants, i.e. 0.007 and 0.008%, respectively, in *A. roxburghii* and 0.005 and 0.013%, respectively, in *A. formosanus*. The content of essential oil

compounds differed significantly between control plants and treated plants with *Bacillus subtilis* 87, i.e. 0.11 and 0.30%, respectively, in *A. roxburghii* and 0.18 and 0.21%, respectively, in *A. formosanus*.

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