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Comparative study for some natural products between two species of *Anoectochilus* genus tissue culture and wild

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Abstract:

Anoectochilus genus is epiphytic Orchid used as traditional medicine. Chemical components and pharmacology have been studied in recent 15 years. Medicinal orchid, in general, is not subjected to detailed pharmacological studies. The aim of this study was to estimate and compare the concentration of bioactive compounds between wild and *in vitro* propagated *Anoectochilus roxburghii* and *Anoectochilus formosanus*. A wide range of chemical compounds are presented including flavonoids, steroids and oil violate which have been isolated recently from these species. Extract and metabolite of these plants, particularly from whole plant , possess useful pharmacological activities .A comprehensive account of chemical constituents and biological activities is presented and a critical appraisal of the ethno pharmacological issues . these species orchid have been empirically used for treatment of different diseases . The results show that the flavonoid, steroid and essential oil contents in three fields were (tissue culture ,after three months and after 6 months in wild) for each species which regarding tissue culture (flavonoid ,steroids and oil essential) In *A.roxiburghii*, the flavonoid contents were (2%,0.9%,6.5%),steroids contents(0.003%,0.007%.0.01%) and the oil essential contents (0.05%,0.3%,0.18%) where as for *A.formosanus* flavenoid contents (6%, 1.05%,6%) steroids contents(0.2,0.005,0.2)% and the oil essential contents (0.1,0.18,0.17)%

Key words : *A.roxiburghii* , medicinal plant, traditional uses, chemical constituents, *Anoectochilus formansus.*

Introduction:

The genus *Anoectochilus* (Orchidaceae family) consists of approximately 40 species distributed from India, the Himalayas, Southeast Asia, and Indonesia to New Caledonia and Hawaii (1).The species *Anoectochilus roxburghii* is used for medicinal and ornamental purposes in China and other Asian countries(2). Because of its unique medicinal properties, such as its effects in clearing heat and cooling blood, eliminating dampness, and detoxification has been called“the king of medicines” (3,4,5,6). Recent research has demonstrated that the entire plant possesses medicinal properties, such as antioxidant, anti-inflammatory and antitumor activities (7, 5,6) The habitat of this species is terrestrial, and the whole plant is used to treat tuberculosis (8). While another study was talking about the essential oil from *A.roxburghii*;to study and investigate the essential oil effect hyperplasy inhibit activity toward the human cell lung cancer cell line so, the essential oil can inhibit the proliferation and increase the apoptosis of the human NCI-H446 tumor cells in vitro(9). as a treatment for liver disease (10) ,and as a treatment for nephritis (11).there is study clarity ,evaluated a novel use of the traditional Asian herb *Anoectochilus formosanus* ,this species is a traditional food item ,generally used for the treatment of liver disorder ,hepatitis ,diabetes ,cardiovascular disorder, etc(12),. *A. roxburghii* is also an expensive ornamental plant that

displays a network of colorful venation in its gorgeous leaves. Although, the flowers are not large, their white labella are quite prominent and toothed, such that a grouping of three or four flowering plants makes an attractive, unique addition to one's orchid collection (13) *A. roxburghii* is now facing extinction because of the destruction of its habitat, the heavy exploitation of its wild resources, its low propagation rate and its slow growth (14,15). Therefore, to meet the growing demand of the herbal and pharmaceutical industries, artificial cultivation is beginning to be investigated and attempted. *A. roxburghii* is usually propagated sexually by seeds. However, the conventional method of propagation (16,5,6). and there is study demonstrate an improved and efficient propagation system, followed by shoot bud induction, shoot proliferation, rooting and acclimatization. Such a protocol would allow for large-scale propagation to meet commercial demand and conserve this threatened orchid species by reducing wild collection. Several protocols for *A.roxburghii* micropropagation have been tested but none has provided an effective method for large-scale micropropagation. The study investigates an improved and efficient propagation method including shoots generation and formation, shoot proliferation, inducing root and acclimatization.(17).another study showed that RSM was successfully applied for SFE optimization of the essential oils from *A.roxburghii*. The pressure and CO2flow rate had significant effect on essential oils yield produced by SFE(5,6).

There are many studies to tissue culture for this genus referred it such as a study was conducted to determine the optimum medium for adventitious buds Consequently, many studies have tried to find methods to increase proliferation and enhance the growth of these plants to protect them from extinction and promote their use in clinical applications. Previous studies have used tissue culture for this genus to determine the optimum medium for adventitious buds (18). Many studies have focused on the tissue culture conditions for this species because natural sources of *A. roxburghii* are depleted (19,20).A. formosanus Hayata belongs to the genus of *Anoectochilus* (family Orchidaceae). After surface sterilization, aboveground parts of *A. formosanus* were collected from Fujian Province, China, and stems with axillary buds measuring about 1 cm were cut into small pieces and cultured in sterile micro propagation medium (MM) containing Murashige and Skoog (21) .

Recently, liquid culture has been considered as an alternative approach to plant micropropagation. Embryogenesis and organogenesis have been performed using large-scale liquid culture system with high automatic level (22). Due to homogenous liquid medium, explants can uptake nutrients at constant concentration in any position on/in the medium. Besides, some modern techniques including shaken culture and bioreactor have been also utilized to upgrade this system. Cell suspension, somatic embryos, bulblets, corms, microtubers or shoot have been cultured in liquid suspension in bioreactors. However, it is showed that some shoots which do not immerge in the water are still seriously vitrificated (23,24).*A.roxburghii* is one of the original plants of the precious natural drugs and is widely medical used, because of great excavation its chemical components and pharmacological activity was well known gradually and the wild resource was in danger. Recently ,adopting biotechnology is regarding a way to breed this species there was the differences of activity constituents between tissue cultured species and wild species. *Anoectochilus* was analyzed and compared between two species for this genus. Results were as follows: HPLC rapid test of flavonoid aglycones was established ,two kinds of flavonoid aglycones from *A. formosanus* were detected simultaneously, the separation accuracy was high and the peak time was early by this method, the total flavonoids contents of two *A. formosanus* after cultivating5months were higher than that of4months in cultivation stage. In the transplant stage, the content of

total flavonoids reached the maximum after4months (in early July).(25)the total flavonoids contents of two *A.formosanus* after cultivating5months were higher than that of 4 months in cultivation stage, in the transplant stage, the content of total flavonoids reached the maximum after4months (in early July) and it was more than wild *A.roxburghii* (8).

Different doses of kinsenoside, a high yielding constituent from *A. roxburghii*, was orally administered to further investigate its biological activity and pharmacological mechanisms that involve in the hypoglycemic effect on streptozotocin (STZ) diabetic rats. This compound exhibited significantly antihyperglycemic activity at the dose of 15 mg/kg body weight.(26)

. These products from this plant are medical uses some we refer to it before in another study clarified three new 3-hydroxybutanolide derivatives, named kinsenbenol and kinsendioside A (27,28), kinsendioside B (29),kinsendiosides A and B were a mixture and one new flavonoid glucoside, named roxburoside (30), were isolated from *A.roxburghii*. the present work aimed (i) to collect *Anoectochilus roxburghii and Anoectochilus formosanus* from different habitats, (ii) *in vitro* propagation of *Anoectochilus roxburghii and Anoectochilus formosanus* under laboratory conditions, and (iii) estimation and comparison of active constituents between micropropagated and wild plants from different habitat.

Materials and Methods:

1 Plant materials

A. roxburghii and *A.formosanus* explant grown on improved MS medium [11], which was supplemented with 3% (w/v) sucrose, and 0.75% (w/v) agar, pH 5.8. explants were grown in a flasks media with a 12-h light/12-h dark photoperiod at(24±1)°C. the whole plants of *Anoectochilus roxburghii and Anoectochilus formosanus* were collected from different natural habitats such as tissue culture ,after 3months and after 6 months in wild.

1.1 Propagation *A. roxiburghii* and *A.formosanus* in tissue culture and greenhouse experiments

1.1.1 Tissue culture

The explants of two species of *Anoectochilus* genus were .*A. roxiburghii* and *A. formosanus*, were cultured for protocorm induction in MS medium containing macronutrients, micronutrients, Ca, organic matter, Fe, 3% sucrose, and agar. The pH was adjusted to 5.8-6.2 with NaOH before autoclaving at 121°C for 15 min and supplemented with different growth regulators. For *A.roxburghii* used 7 media and for another species used 4 media to propagate these species were used for tissue culture growth .After autoclaving, the media were left in the culture room for approximately 14 days, then 4-5 explants were cultured in each flask. These cultures were maintained at 25 ± 2°C with a 12 h/12h light/dark cycle at 25 ± 2°C.

1.1.2 greenhouse experiments

Experiments were designed under greenhouse conditions using the micropropagation of *A. roxiburghii* and *A.formosanus*. After90days and 180 days of micropropagation in *vitro*, aseptic plants were grown in a greenhouse in8 cm × 8 cm × 9 cm plastic pots containing 1 kg of a peat moss/humus soil mixture at a ratio of 1:1 then mixed with the upper soil surface in each pot. Other *A. roxiburghii* and *A.formosanus* plants were planted separately in sterile soils. 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.

1.2Extraction and Samples Preparation for *A.roxburghii* and *A.formosanus* and isolation flavonoids contents

0.1g whole plants powder in 4ml of cold 70 ethanol .The homogenate was centrifuged at 12000 speed for 5min at 4c and the supernatant was used to measure intracellular contents.the determination of flavenoids contents followed the methods of jia and Eberhardt with modification ().the supernatant diluted 5 times with 70% ethanol(that means 1ml of supernatant with 4ml of 70 ethanol). Take rutin solution according to concentrations which are (0.2, 0.4, 0.6, 0.8 ,1.0), then each tube add to them0.3ml of 5%NaNo2, 1ml of 70%ethanol mixed together and set aside for 6 min; then 0.3 ml of 4%Al(NO3)3 was added and set aside for 6 min, 2ml of 4%NaOH was added after 10 min was measured at 510nm.then repeat this procedure also to *A.roxiburghii* tissue culture through 3 months and 6 months in fields and comparative between them and tissue culture for *Aformosanus* and also in different hapitate.

1.3 Measurement of intracellular and extracellular total steroids contents for *Anoectochilus roxiburghii and A.formasanus*

weigh 2g of plant materials fresh in a 100ml blue cap bottle and add magnetic stirring son at the same time, then add 75 ml of n-hexane and magnetic stir for 10 min. followed with 30min of ultrasonic extraction, then pour the supernatant into two 50ml centrifuge tubes after standing. add 20ml of n-hexane to the residue, after magnetic stirring for 5 min, merge the supernatant into centrifuge tube, and 5000r/min centrifuge for 10 min. Pour the supernatant into mouth round bottom flask and concentrate it to dryness at 40℃ by rotary evaporation. Evaporation residue was added 25 ml of ethanol, 5ml KOH solution (500 g / L).then reflux for 30 min with boiling water bath. Then cooling to room temperature with running water immediately after the reaction. Transfer the saponification solution to a 200ml separator funnel (Teflon stopcock).then wash the round bottom flask with 10ml of deionizer water and merge the lotion into the separator funnel, then extract for three times with 50、50、30ml of petroleum ether (the boiling point is 30-60 ℃） respectively. merge the three organic phase into 200ml separator funnel(Teflon stopcock),add 30ml of de ionized water and oscillate for 2 min ,then discharge the lower aqueous layer and measure its PH .wash the teflon stopcock with deionizer water repeatedly until it is neutral. Add 3-5g anhydrous sodium sulfate to the extract and oscillate for 2 min. then add the extract to 100ml round bottom flask and concentrate it to dryness by rotary evaporation, then can get un saponifiable matter, dissolve it with glacial acetic acid and dilute to 5ml, as the sample solution.

Preparation of standard curve and colorimetric detection:

1. Preparation of color-substrate solution: chill the acetic anhydride and concentrated sulfuric acidat 4 ℃ refrigerator for more than 2 hours first, then take a 150ml dry grinding mouth bottle and place it in an ice water bath. Add 60 ml of acetic anhydride, 30 ml of glacial acetic acid, and then slowly add 10 ml of concentrated sulfuric acid, mix well. Then add 1g of anhydrous sodium sulfate, store at 4 ℃ for later use.

2. Choice of wavelength :Take 5 ml color-substrate solution in a 12ml dry clean glass vial with a precision pipette, then add 200µl sample solution, tighten the screw cap, after coloration at 23 ℃ water bath for 20min, determine the maximum absorption wavelength

through UV scanning in the range of 190 ~ 900nm within 5min (Color will change over time, so it should be tested as soon as possible).

3. Preparation of standard substance: weigh accurately 20 mg in 1 ml of acitic acidβ-Sitosterol standard , and get a series of standard solution through method of step wise dilution. The solvent is acetic acid ,the concentration are 0.01、0.03、0.05、0.07、0.1、0.12、0.15 mg /ml respectively ,as samples, measure their absorbance according to the step 2 and draw standard curve.289nm

4-4. Sample testing :Take sample solution under test and test it according to the step 2, then measure the content of sterol according to the absorbance that have got.

1.4Extraction of the oil essential

take at least 10g which culture in field 50g from tissue culture fresh material, and wash with water to remove the dirties , then cut them into pieces .Put the sample into cycle ground bottle and add the water to cover the sample .Put the bottles with sample on a Reflux device, and Distill the essential oil at 100C(heating with the electric jacket by setting the temperature at 200C).Put 5 ml ether solute into the receiving tube when steam were seen in the "condensation tube"Collect(the ether and oil) using anew flask after 30 min. Add another 5 ml ether solute into "receiving tube" and collect(the ether and oil" after 20min .Repeat step 6 many times for 5 h. and prepare the NaCl solution(108g in 300 ml of distilled water)in the spare time. Make the volume of the water layer and the ether layer equal. Add NaCl solution the collected solution (ether and oil) and shaking for two min then put stand for several time to make two layer. Collect the up layer into anew flask using separator funnel. Prepare a chromatographic column using sodium anhydrous sulfat. Dry the collected solution in step 10 ,and collected the outflow liquid into a 100 ml Distilling round flask (if the volum more than 50 ml using a150 ml Distilling round flask) and evaporated it with evaporate rotary.
When the volum decreased to 25 ml ,transfer the solution to appear shaped flask (weight the empty flask firstly) and continue the evaporation. Measure the weight of the flask again

Results:

Table1:effect hormones on *Anoectochilus roxiburghii* tissue culture growth

	NAA	2,4D	ZT	6BA	KT	IBA	growth
M1	**0.5**	0.5		1	0.25		+
M2	**1**	1		0.5	0.5		+
M3	**0.9**	0.2	0.25	1			++
M4	**1**	0.1		3	0.5	0.04	++
M5		0.1		1			+
M6	**0.5**			3			_
M7	**2**		1			2	_

Note:+ ⟶ good growth ,++ ⟶ very good growth

Table2: effect hormones on *Anoectochilus formasanus* tissue culture growth

	NAA	KT	6BA	2,4D	IBA	growth
M1	1	0.5	3	0.1	0.04	+
M2	0.2		4			+
M3	1	0.5	0.5	1		_
M4	1		2			_

In tables(1,2) there are many media have different hormonal concentrations use it to propagation *Anoectochilus roxiburghii* and *A.formosanus* and the results show the (M3,M4)is the best media to propagation *A.roxiburghii* while (M1,M2) regard the best medium for *A .formosanus* and the rest media the growth is very slowly .

There are results showed that the different ratios from flavonoid contents where (2%,6%) for *Anoectochilus roxiburghii* and *A.formosanus* tissue culture respectively from other periods while the steroids contents were 0.2% in the *A.formosanus* higher from in *A.roxiburghii* which were between 0.003-0.01% and With respect to oil essential contents were (0.05%,0.3%,0.18%) for *A.roxiburghii* while the oil essential in *A.formosanus* were (0.1,0.18,0.17) respectively (Figure2A.B).

In this study refers to the oil essential contents and comparative between three periods in tissue culture , after 3months and after 6 months in the (figure 3A) the results show the oil essential contents were fatty acids , Acid esters , Alkanes ,Esters,Olefins, Alcohols, Ketones ,Carboxylic acid derivatives for *A. roxburghii* and in the (figure3B) The oil essential compounds for *A.formosanus* contain Aldehyds in addition for that compounds in the oil essential for *A roxburghii* .

A1 A2 A3

B1 B2 B3

FIG1:-different periods for two species

A:*Anoectochilus roxiburghii*(A1. tissue culture,A2.after3months ,A3. after 6months)

B:*Anoectochilus formsansus*(B1. tissue culture ,B2.after3months,B3. after 6 months)

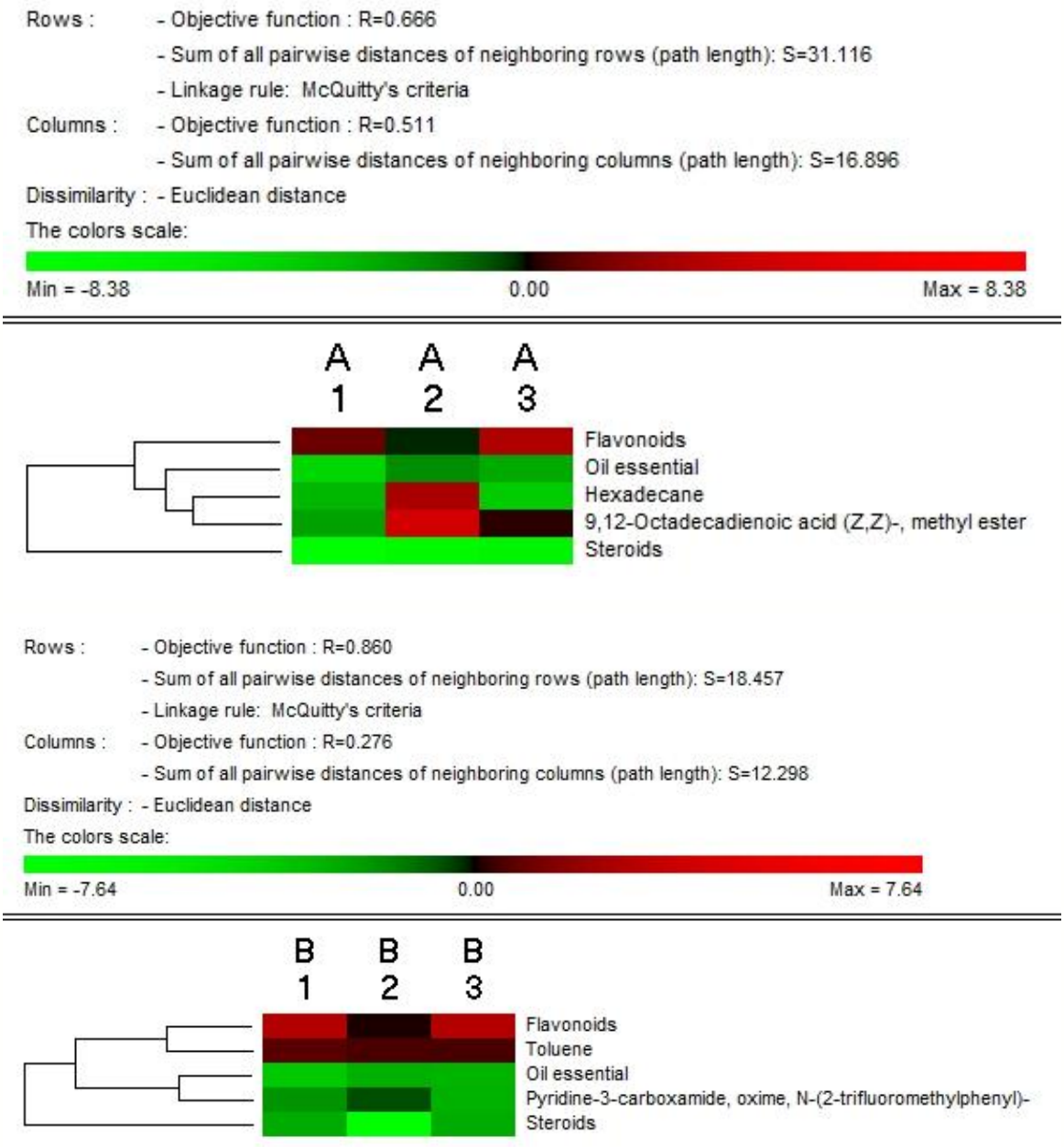
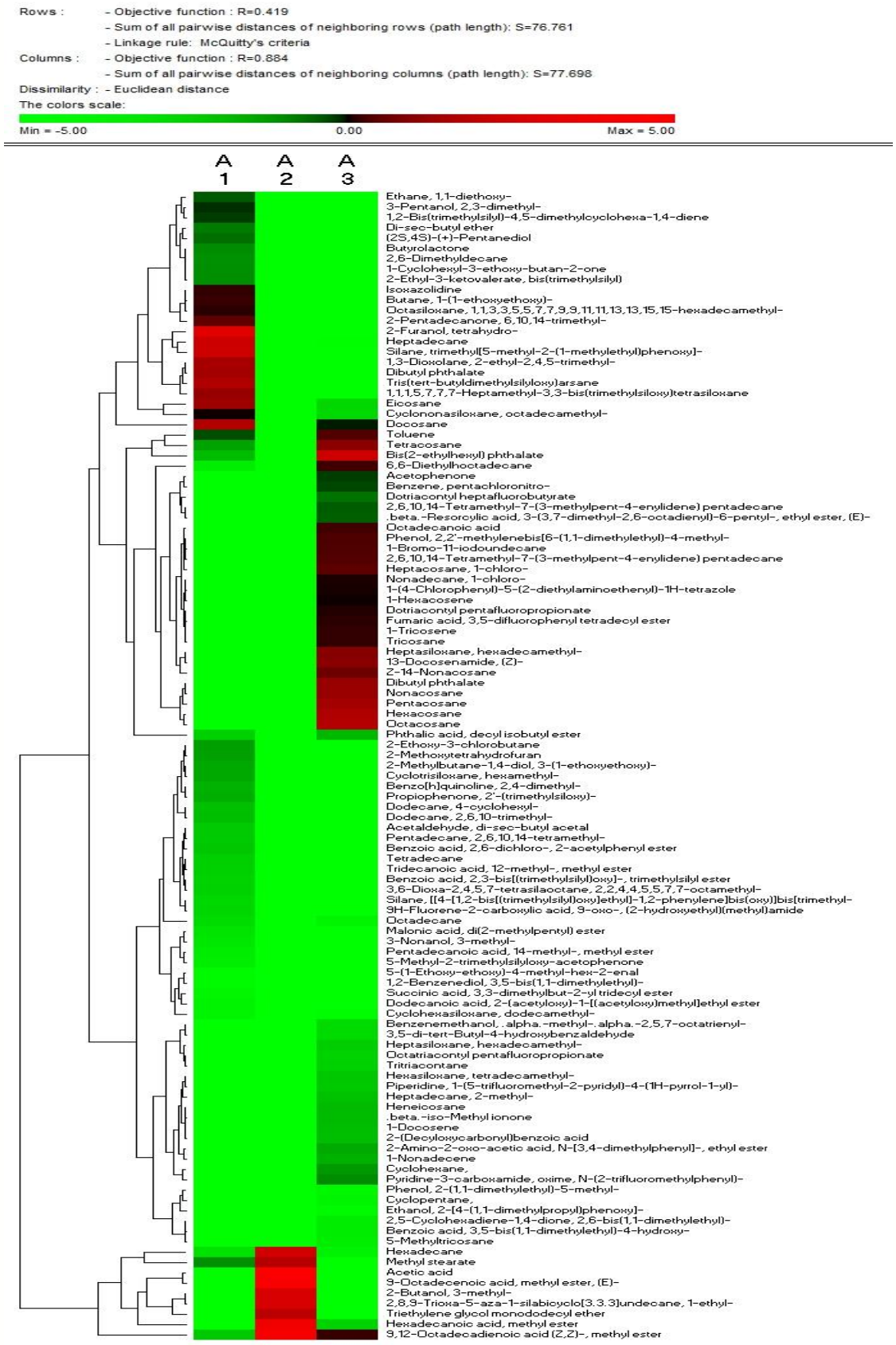


Fig 2 A-Comparative Analysis of components between *Anoectochilus roxiburghii* A1-tissue culture , A2 -after 3 months , A3-after 6 months

B- Comparative Analysis of components between *Anoectochilus formosanus*B1-tissue culture , B2 -after 3 months , B3-after 6 months.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- Sum of all pairwise distances of neighboring rows (path length): S=76.761
- Linkage rule: McQuitty's criteria
Columns : - Objective function : R=0.884
- Sum of all pairwise distances of neighboring columns (path length): S=77.698
Dissimilarity : - Euclidean distance
The colors scale:
Min = -5.00
0.00
Max = 5.00
A 1
A 2
A 3
Ethane, 1,1-diethoxy-
3-Pentanol, 2,3-dimethyl-
1,2-Bis(trimethylsilyl)-4,5-dimethylcyclohexa-1,4-diene
Di-sec-butyl ether
(2S,4S)-(+)-Pentanediol
Butyrolactone
2,6-Dimethyldecane
1-Cyclohexyl-3-ethoxy-butan-2-one
2-Ethyl-3-ketovalerate, bis(trimethylsilyl)
Isoxazolidine
Butane, 1-(1-ethoxyethoxy)-
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-
2-Pentadecanone, 6,10,14-trimethyl-
2-Furanol, tetrahydro-
Heptadecane
Silane, trimethyl[5-methyl-2-(1-methylethyl)phenoxy]-
1,3-Dioxolane, 2-ethyl-2,4,5-trimethyl-
Dibutyl phthalate
Tris(tert-butyldimethylsilyloxy)arsane
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane
Eicosane
Cyclononasiloxane, octadecamethyl-
Docosane
Toluene
Tetracosane
Bis(2-ethylhexyl) phthalate
6,6-Diethylhoctadecane
Acetophenone
Benzene, pentachloronitro-
Dotriacontyl heptafluorobutyrate
2,6,10,14-Tetramethyl-7-(3-methylpent-4-enylidene) pentadecane
.beta.-Resorcylic acid, 3-(3,7-dimethyl-2,6-octadienyl)-6-pentyl-, ethyl ester, (E)-
Octadecanoic acid
Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-
1-Bromo-11-iodoundecane
2,6,10,14-Tetramethyl-7-(3-methylpent-4-enylidene) pentadecane
Heptacosane, 1-chloro-
Nonadecane, 1-chloro-
1-(4-Chlorophenyl)-5-(2-diethylaminoethenyl)-1H-tetrazole
1-Hexacosene
Dotriacontyl pentafluoropropionate
Fumaric acid, 3,5-difluorophenyl tetradecyl ester
1-Tricosene
Tricosane
Heptasiloxane, hexadecamethyl-
13-Docosenamide, (Z)-
Z-14-Nonacosane
Dibutyl phthalate
Nonacosane
Pentacosane
Hexacosane
Octacosane
Phthalic acid, decyl isobutyl ester
2-Ethoxy-3-chlorobutane
2-Methoxytetrahydrofuran
2-Methylbutane-1,4-diol, 3-(1-ethoxyethoxy)-
Cyclotrisiloxane, hexamethyl-
Benzo[h]quinoline, 2,4-dimethyl-
Propiophenone, 2'-(trimethylsiloxy)-
Dodecane, 4-cyclohexyl-
Dodecane, 2,6,10-trimethyl-
Acetaldehyde, di-sec-butyl acetal
Pentadecane, 2,6,10,14-tetramethyl-
Benzoic acid, 2,6-dichloro-, 2-acetylphenyl ester
Tetradecane
Tridecanoic acid, 12-methyl-, methyl ester
Benzoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester
3,6-Dioxa-2,4,5,7-tetrasilaoctane, 2,2,4,4,5,5,7,7-octamethyl-
Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)]bis[trimethyl-
9H-Fluorene-2-carboxylic acid, 9-oxo-, (2-hydroxyethyl)(methyl)amide
Octadecane
Malonic acid, di(2-methylpentyl) ester
3-Nonanol, 3-methyl-
Pentadecanoic acid, 14-methyl-, methyl ester
5-Methyl-2-trimethylsilyloxy-acetophenone
5-(1-Ethoxy-ethoxy)-4-methyl-hex-2-enal
1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-
Succinic acid, 3,3-dimethylbut-2-yl tridecyl ester
Dodecanoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl]ethyl ester
Cyclohexasiloxane, dodecamethyl-
Benzenemethanol, .alpha.-methyl-.alpha.-2,5,7-octatrienyl-
3,5-di-tert-Butyl-4-hydroxybenzaldehyde
Heptasiloxane, hexadecamethyl-
Octatriacontyl pentafluoropropionate
Tritriacontane
Hexasiloxane, tetradecamethyl-
Piperidine, 1-(5-trifluoromethyl-2-pyridyl)-4-(1H-pyrrol-1-yl)-
Heptadecane, 2-methyl-
Heneicosane
.beta.-iso-Methyl ionone
1-Docosene
2-(Decyloxycarbonyl)benzoic acid
2-Amino-2-oxo-acetic acid, N-[3,4-dimethylphenyl]-, ethyl ester
1-Nonadecene
Cyclohexane,
Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-
Phenol, 2-(1,1-dimethylethyl)-5-methyl-
Cyclopentane,
Ethanol, 2-[4-(1,1-dimethylpropyl)phenoxy]-
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-
Benzoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-
5-Methyltricosane
Hexadecane
Methyl stearate
Acetic acid
9-Octadecenoic acid, methyl ester, (E)-
2-Butanol, 3-methyl-
2,8,9-Trioxa-5-aza-1-silabicyclo[3.3.3]undecane, 1-ethyl-
Triethylene glycol monododecyl ether
Hexadecanoic acid, methyl ester
9,12-Octadecadienoic acid (Z,Z)-, methyl ester

A

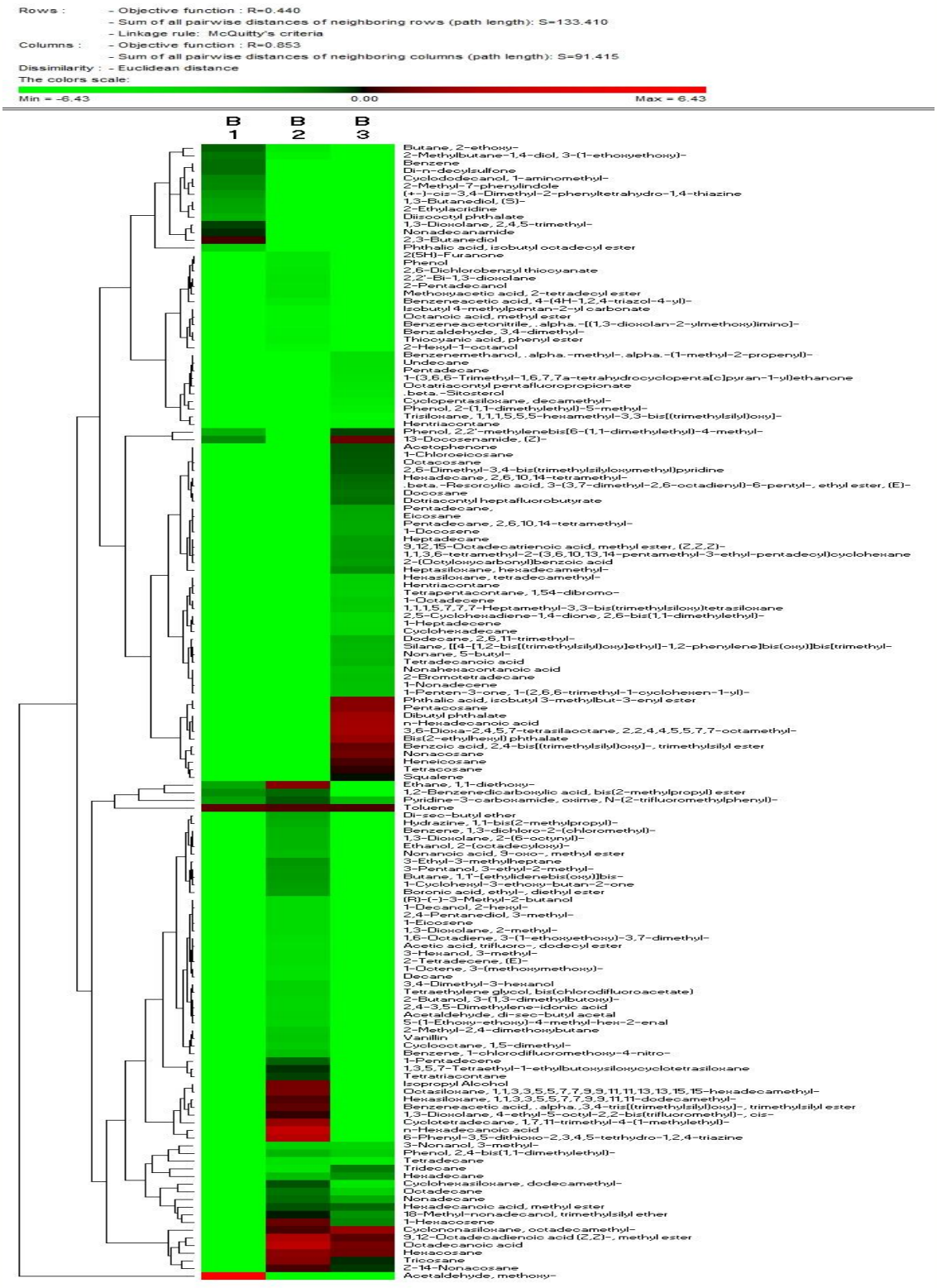

Fig 3 A-Comparative Analysis of Oil essential components between *Anoectochilus roxiburghii* A1-tissue culture , A2 -after 3 months , A3-after 6 months
B- Comparative Analysis of Oil essential components between *Anoectochilus formosanus*B1-tissue culture , B2 -after 3 months , B3-after 6 months.

Colors in the heat map mean the fold change in according to the above, red and green represent the higher and lower levels, respectively.

Discussion:

There is study refer to the tissue culture conditions of *Anoectochilus roxburghii.* For disinfection effect on the explants and for the best bud proliferation medium could improve bud proliferation and culture of strong seedlings. (31)while there was another study refer to culture medium for *Anoectochilus formosanus* cultural seedling rooting culture were studied with orthogonal experimental (32), and another study clarify that TDZ is now being widely used for micropropagation of several plants including many orchids because of its tremendous ability to induceorganogenesis (33, 34, 35, 36) In view of the fact that these plants are endangered and demonstrate slow growth and sensitivity to various pathogens, it is necessary to find ways to protect, increase the growth, and enhance the yield of medicinal products from these plants.

In this study, we showed the effect of different hormone concentrations on *A. roxburghii* and *A. formosanus* growth in tissue culture experiments. M3 and M4 were the best media for the propagation of *A. roxiburghii* plants. M3 contained growth regulators (NAA 0.9, 2,4D 0.2, ZT 0.25 ,6-BA 1mg /L), as did M4 (NAA 1, 2,4D 0.1, 6-BA 3, KT 0.5, IBA 0.04 mg/L) while the best medium for the propagation of *A. formosanus* was M4, the same as for *A. roxiburghii*.

The aboveground parts of *A. formosanus* were collected from Fujian Province, China. Stems with axillary buds measuring about 1 cm were cut into small pieces and cultured in sterile micropropagation medium (MM) containing Murashige and Skoog (21) basic medium supplemented with 1-naphthaleneacetic acid (NAA), 6-benzyl adenine (6-BA), activated charcoal, and sucrose. The medium was solidified with agar and then incubated at 25±2°C under a 12 h photoperiod (37), for A roxburghii TDZ is sometimes associated with morphologicalabnormalities as has been reported in several species (38).

The results show that the flavonoid, steroid and essential oil contents in three fields were (tissue culture ,after three months and after 6 months in wild) for each species which regarding tissue culture (flavonoid ,steroids and oil essential) In *A.roxiburghii*, the flavonoid contents were (2%,0.9%,6.5%),steroids contents(0.003%,0.007%.0.01%) and the oil essential contents (0.05%,0.3%,0.18%) where as for *A.formosanus* flavenoid contents (6%, 1.05%,6%) steroids contents(0.2,0.005,0.2)% and the oil essential contents (0.1,0.18,0.17)%, the results show the oil essential contents were fatty acids , Acid esters , Alkanes ,Esters,Olefins, Alcohols, Ketones ,Carboxylic acid derivatives for *A. roxburghii* and in the (figure3B) The oil essential compounds for *A.formosanus* contain Aldehyds in addition for that compounds in the oil essential.

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