Scientific Research Publishing

Advances in Bioscience and Biotechnology, 2016, 7, 418-427
http://www.scirp.org/journal/abb
ISSN Online: 2156-8502
ISSN Print: 2156-8456

Micropropagation of *Curcuma Sp*., a Threatened Medicinal Plant

Phan Van Tan

Faculty of Agriculture and Forestry, Taynguyen University, Buonmathuot city, Vietnam
Email: phvtan56@yahoo.com

How to cite this paper: Tan, P.V. (2016) Micropropagation of *Curcuma Sp*., a Threatened Medicinal Plant. *Advances in Bioscience and Biotechnology*, **7**, 418-427.
http://dx.doi.org/10.4236/abb.2016.710040

Received: September 24, 2016
Accepted: October 28, 2016
Published: October 31, 2016

Abstract

A rapid and improved micropropagation protocol was developed for *Curcuma sp*., a threatened and high value medicinal plant, using main bud sprout and top of rhizome sprout as explants. Stepwise optimization of different plant is to change the growth regulator, reduce the level of macro-nutrition and add humate. The present study has created multiple shoot and root induction and plantlet *in vitro* culture, transfers the plantlet to *ex vitro*. The M2 medium (MS's macronutrition ¼, MS's micronutrition + Morel's vitamine + coconut milk 10% + sucrose 25 g/l + humate 1.0 ml/l + agar 7.5 g/l + 2,4D 0.5 mg/l + BAP1.0 mg/l + TDZ 1.0 mg/l) is the highest ratio of callus induction. The TA3 medium (MS's macronutrition ¼ + MS's micronutrition + Morel's vitamin+ coconut milk 10% + sucrose 25g/l + humate 2.0 ml/l + 2,4 D 0.5 mg/l + Kinetin 2.0 mg/l + TDZ 1.0 mg/l + BAP 1.0 mg/l2 + NAA 2.0 mg/l + activated carbon 2.0 g/l) is able to create buds and regeneration multiple bud for *Curcuma sp*. TA3 medium adding IAA 2 mg/l and IBA 0.5 mg/l has resulted in the highest indices of quantity, healthy shoot and large diameter of roots. A large number of healthy plantlets are induced by the medium of MS's macronutrients ½, MS's micronutrients full, Morel's vitamin, humate 3 g/l, coconut milk 150 ml/l, activated carbon 3 g/l, composition phytohormone: IAA 2 mg/l + BAP 2 mg/l + TDZ 0.5 mg/l. Further studies should focus on optimizing the humate concentration for these species of Zingiberaceae. The duration of the research is from 5/2015 to 4/2016 at Faculty of Agriculture and Forestry, Tay Nguyen University, Vietnam.

Keywords

Curcuma Sp., Humate, *In Vitro* Culture, Multiple Bud, Shoot Multiplication

1. Introduction

Poganggan (*Curcuma sp*., Zingiberaceae family, Zingiberalesordo) is the medicinal species, and has high value use in medicine. Many local people in Tay Nguyen (moun-

tain area), Vietnam have been using it. We have not determined the science name of its species, because its inflorescence was not found but its gene had close ties with *Curcuma vitellina* by DNA [1].

Curcuma sp. has been known to contain higher polyphenol, alkalloid, saponin than other species in the same genus and is good for heart operation and circulatory system, improving the people's health [1].

In nature, *Curcuma sp.* has low reserve and is distributed in very narrow area in Kon Bong 2 village, Dakrong commune, Kbang district, Gialai province, Vietnam. It needs to be conserved and cultured. However, natural regeneration is rather limited and needs tissue culture to micropropagation. So, it needs to be propagated by tissue culture. We have used medium MS (Murashige and Skoog, 1962) [2], but if we only use inorganic minerals in supply nutrition, then its tissue could not have division cell to become callus or propagate the root and shoot. We have improved the micropropagation protocol: reducing the level of macro-nutrition and phytohormon, adding humate, and we have had the successful outcome, which is able to create multiple shoot and root induction and plantlet *in vitro* and move plantlet to *ex-vitro*. In order to make plantlet *in vitro*, we need to proceed below steps: 1) select the organs to culture tissue and its sterilized methods; 2) select the suitable culture medium; 3) select the medium and phytohormone for buds forming *in vitro*; 4) select medium and phytohormone for root induction *in vitro*; and 5) build plantlets *in vitro*. The duration of research is from 5/2015 to 4/2016 at Faculty of Agriculture and Forestry, Tay Nguyen University, Vietnam.

2. Materials and Methods

2.1. Plant Materials

Poganggan (*Curcuma sp.*) are collected from native places: Kon Bong 2 Village, Dakrong commune, Kbang district, Gialai province, Vietnam.

2.2. Research Methods

Every research item, mentioned above, is the experiment; every experiment was Randomizid Complete Block Design (RCBD), 3 repetitions; the data was from 30 to 50 replicates per treatment.

2.2.1. Sterilization Methods

Sterilization solution is NaClO, 3 levels of concentration: 0.5%, 1.0%, 1.5%; processing time: 7, 10, 15 minutes; repeated 3 times; 50 samples for each times; taking samples: main bud sprout, top of rhizome sprout and shoot segments.

Sample after sterilization is implanted into the glass bottle (V = 250 ml) containing the macro and micro nutrition MS's medium (Murashige and Skoog, 1962) [2], Morel's vitamin (1951) [3], added sucrose 20 g/l, agar7.5 g/l, pH level is 5.8.

2.2.2. Select the Suitable Culture Media

Experiment 1: *Select the media which have the ability to regenerate the cell.*

M0 (control)	MS's macro and micro nutrition + Morel's vitamine + coconut milk 10% + sucrose 25 g/l + agar 7.5 g/l + 2,4D 0.5mg/l + BAP1.0 mg/l + TDZ 1.0 mg/l
M1	MS's macro nutrition ½, MS's micro nutrition + Morel's vitamine + coconut milk 10% + sucrose 25 g/l + agar 7.5 g/l + 2,4D 0.5 mg/l + BAP1.0 mg/l + TDZ 1.0 mg/l
M2	MS's macro nutrition ¼, MS's micro nutrition + Morel's vitamine + coconut milk 10% + sucrose 25 g/l + humate 1.0 ml/l + agar 7.5 g/l + 2,4D 0.5 mg/l + BAP1.0 mg/l + TDZ 1.0 mg/l
M3	MS's macro nutrition 0, MS's micro nutrition + Morel's vitamine + coconut milk 10% + humate 1.0 ml/l + sucrose 15 g/l + agar 7.5 g/l + 2,4D 0.5 mg/l + BAP1.0 mg/l + TDZ 1.0 mg/l
M4	$Ca(NO_3)_2$ 800 mg/l + KH_2PO_4 170 mg/l + MS's micro nutrition 1/2 + Morel's vitamine + coconut milk 10% + humate 1.0 ml/l + sucrose 25 g/l + agar 7.5 g/l + 2,4D 0.5 mg/l + BAP1.0 mg/l + TDZ 1.0 mg/l

2.2.3. Select Media for Shoot Multiplication

Experiment 2: *Select media for shoot multiplication.*

TA1	$Ca(NO_3)_2$ 800 mg/l +KH_2PO_4 170 mg/l + MS's macro nutrition 0 + MS's micro nutrition + Morel's vitamin +coconut milk 10% + sucrose 25g/l + humate 3.0ml/l + IBA 2.0 mg/l + IAA 2.0 mg/l + Kinetin 2.0 mg/l + TDZ 1.0 mg/l + BAP 2.0 mg/l
TA2	MS's macro nutrition¼ + MS's micro nutrition + Morel's vitamin+coconut milk 10% + sucrose 25g/l +humate 2.0ml/l + 2,4D 0.5mg/l + IBA 3.0 mg/l + IAA 2.0 mg/l + Kinetin 2.0 mg/l + TDZ 1.0 mg/l + BAP 1.0 mg/l
TA3	MS's macro nutrition ¼ + MS's micro nutrition + Morel's vitamin+coconut milk 10% + sucrose 25g/l + humate 2.0 ml/l + 2,4D 0.5 mg/l + Kinetin 2.0 mg/l + TDZ 1.0 mg/l + BAP 1.0 mg/l2 + NAA 2.0 mg/l + activated carbon 2.0 g/l

2.2.4. Select Media for Root Induction

Experiment 3: Select the media for root induction

From the best medium of experiment 2, we continue with the experiment 3, the aim is to get the best root induction. We used 3 plant growth regulators: IAA (indol acetic acid), IBA (indol butyric acid) and NAA (naphthalene acetic acid) with different concentrations. IAA: 0.5; 1.0, 2.0, 3.0 mg/l; IBA: 0.5, 1.0, 2.0, 3.0 mg/l; NAA: 0.5, 1.0, 2.0, 3.0 mg/l; TDZ (thidiazuron) fixedly.

2.2.5. Select the Media for Shoot Multiplication

Experiment 4: Select the media for shoot multiplication

From the result in experiment 3, we got the best recipe can apply to experiment 4; the experiment changes kind and concentration of kinetin or BAP (benzylaminopurine); kinetin: 1.0, 2.0, 4.0, 6.0 mg/l or BAP: 1.0, 2.0, 4.0, 6.0 mg/l.

2.2.6. Select the Media for Plantlet

Experiment 5: Select the media for plantlet

From the best result of experiments 3 and 4, separate the plantlet and move to experiment 5 in order to get the complete plantlet, strong enough to move to *ex vitro*.

Culture medium for fully plantlet base on TA3 medium but some modifications: MS's macronutrients ½, MS's micronutrients, Morel's vitamin, humate 3.0 ml/l, coconut milk 150 ml/l, active carbon 3.0 g/l, NAA 2.0 mg/l, BAP 2.0 mg/l, TDZ 0.5 mg/l, agar 7.5 g/l, pH 5.5-6.0.

2.3. Culture Conditions

Temperature 25˚C ± 0.5˚C, RH% 50% - 60%, brightness 2000 lux, 12 hours of light/day.

2.4. Data Processing

Using EXCEL, Duncan's testis applied to distinguish the difference with/without statistical significance.

3. Results and Discussions

3.1. Sterilization Results

From 3 organs: main bud sprout, top of rhizome sprout (its shoot doesn't have leaves, downward towards land) and rhizome segment length 2 - 3 cm, NaClO 1% concentration is chosen, processing time is 10 minutes and the best organs are main bud sprout and top of rhizome sprout, survival rate 67%.

3.2. Histological Studies

Used the result of micropropagation of the species from the same Curcuma genus: *C. longa* [4] [5], *C. zedoaria* [6], *C. amarrotica* [7], *C. caesia* [8] [9], *C. mangga* [10]-[12], use medium MS (compele-100%) after 4, 6, 8 weeks, the result is not good: samples not division cell, could not form callus. Therefore, we have to test with 5 mediums as **Table 1** in order to choose a suitable medium to cell of *Curcuma sp.* can survive and division cell.

When the sample of tissue culture can survive, the highest ratio of call us induction is M2 (70%). In medium M2, the callus, with normal diameter still have good ability separate need to be selected.

Induction of callus is 2 types of samples: main bud sproutand top ofrhizome sprout, whatcontinue growth to bud is the chance for next step of tissue culture. The medium M2 has the best probable of create bud (50%); every sample can form a bud after 3 weeks **Figure 1(a)**. The difference of between 3 criteria has indicated the statistical significance.

We have selected the medium M2 suit to tissue culture of *Curcuma sp.* as final result. The medium M2 has ratio of only MS's macronutrients ¼, MS's micronutrients 100%, Morel's vitamin and most important add is humate 1 ml/l. In nature *Curcuma sp.* has used only organic material (in humus) and is not suitable for high concentrations of inorganic nutrients. However medium M3 (no macronutrient) and M4 only has Ca, K, P are inappropriate for tissue culture for *Curcuma sp*.

Table 1. The result of the adaptation of *Curcuma sp*. in the medium after 3 weeks.

Sign medium	Callus induction (%)	Diameter callus (mm)	Create bud (%)
M0	4.0	6.0	0.0
M1	30.0	6.5	0.0
M2	70.0	4.8	50.0
M3	14.0	3.6	1.4
M4	28.0	4.0	3.0
CV%	15.11	14.6	3.4
$LSD_{0.05}$	26.0	1.8	28.0

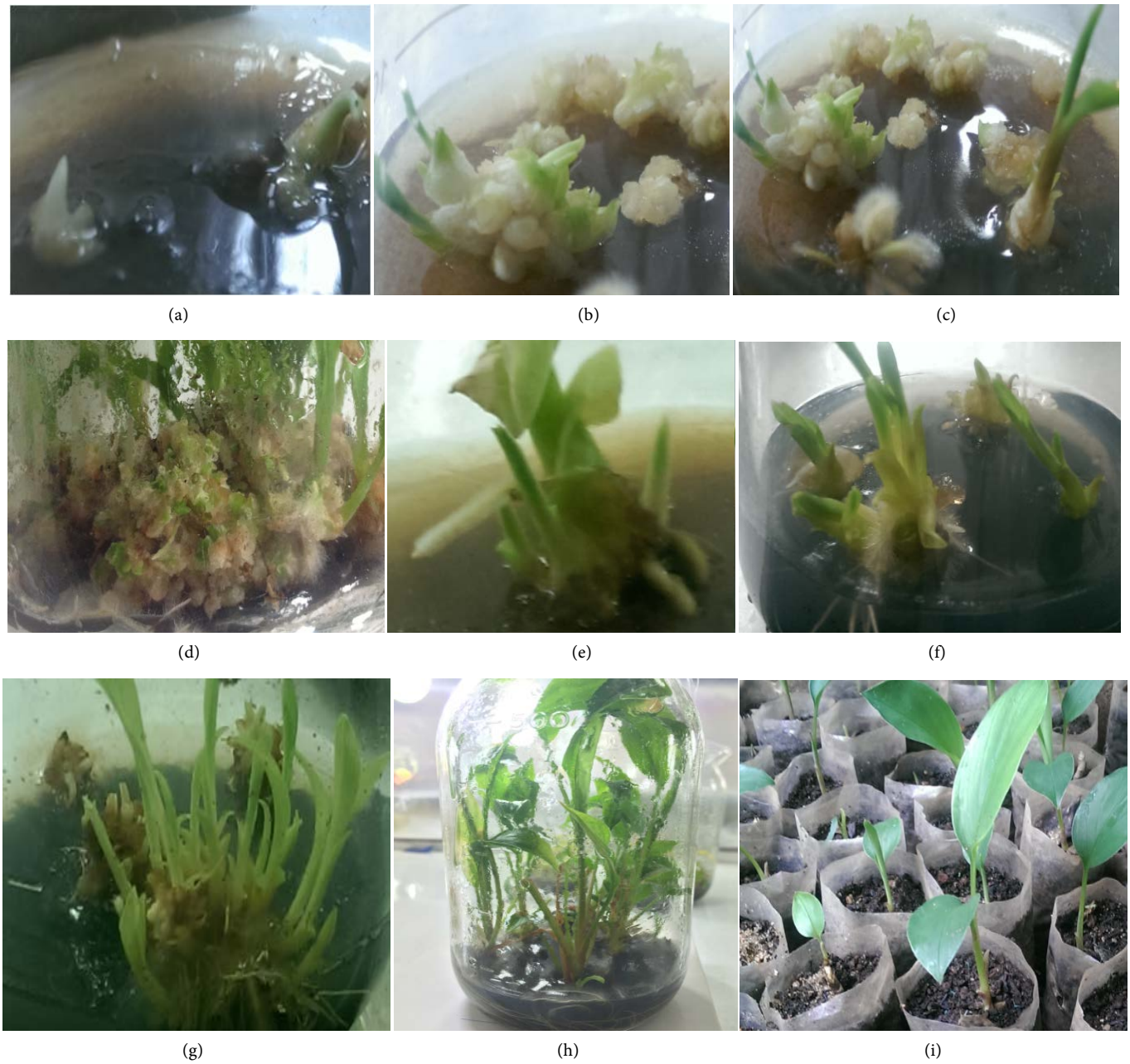

Figure 1. Plant regeneration from callus derived from *in vitro* culture of *Curcuma sp.* (a) new bud derived from main bud sproutand top of rhizome sprout in M2 medium after 3 weeks; (b-d) multiple bud in TA3 medium; (e-f) root forming in TA3 medium what adding IAA 2 mg/l; (g) multiple shoots in medium as TA3 except MS's macro nutrition ½ + MS's micro nutrition + humate 3 g/l + BAP 2 mg/l; (h) plantlet of *Curcuma sp.*; (i) successful plantlet growth after being transferred into the net house.

Select the Media for Propagation via Shoot Morphogenesis

The media in the experiment 2 can be in long time because callus can be lost the morphogenesis. We only culture medium M2 in a short time, only 3 to 4 weeks; then finding the medium for shoot (may have roots or not, or very little roots). *Curcuma sp* belongs to class Monocotyledonae it should be able to form the multiple shoot, increasing the rate of formation of plantlet.

Table 2 with 5 criteria: number buds/samples, bud formation rate/week, bud height, number of leaves/bud and the roots/bud showed medium TA3 is the best medium which can be the base for subsequent experiment Figures 1(b)-(d). This is medium where MS reduces to 1/4, add organic source is sucrose 25g/l, 10% coconut milk, humate 2 ml/l and activated carbon 2g/l. Reduce MS medium to ½ is the same of the result of Sharmin *et al.* [7] [12]. TA1 and TA2 medium have low number of roots and the newly buds is low although the inorganic components have not been. *Curcuma sp.* is probably only suitable with culture medium where reduced inorganic nutrient and must have humate and activated carbon.

TA3 medium has created the new buds with the ratio of buds/sample is 9.2 (Figure 1(d)), outperformed TA1 and TA2 medium (only 3.5 to 5.0 buds/sample). Bud formation rate is the most important indicator in this culture stage; TA3 gives the highest bud formation rate (1.0 bud after 1 week); samples from TA3 medium after 4 weeks had 8 - 10 buds/sample. TA3 medium creates buds in culture of *Curcuma sp.* Shoot regeneration has multiple bud, propagation speed will increase significantly.

The difference between recipes is statistically significant.

3.3. Induction Roots

Using TA3 medium (MS'smacronutrients ¼ + MS's micronutrients + Morel's vitamin + sucrose 25 g/l + Humat 2 ml/l + activated carbon 2 g/l) had bud clusters Figure 1(g), changes the kind and concentrations of auxin group to define the best medium for root forming.

Table 3 shows that all three kinds of auxin can stimulate roots former for *Curcuma sp.*, after 30 days placed in rooting medium, the root forming are successful for all 12 recipes, however, root forming rate is differ between the recipes. After 10 days, the IAA recipes have the rate of new roots higher than IBA and NAA, the highest rate belongs to the IAA 2 mg/l; increasing the concentration of the same kind, root forming ability decreased; with 3 above kinds, the concentrations for root forming are equal or less than 2 mg/l.

The quantity of roots in the bud is more important indicator; *Curcuma sp.* as well as other tissue culture plantlets would rather have few strong roots than high quantity of roots. The strong and fat root will be easier for next culture and move to *ex vitro*. All 12

Table2. Number and size bud of *Curcuma sp.* in medium TA after 4 weeks.

Sign medium	Number buds/sample	Bud formation rate/week	Bud height(cm)	No. of leaves/bud	No. of roots/bud
TA1	3.5	0.3	3.4	2.2	0.0
TA2	5.0	0.7	3.0	2.2	0.8
TA3	9.2	1.0	4.4	2.4	4.6
CV%	5.3	3.5	2.4	1.9	5.2
$LSD_{0.05}$	1.8	0.3	1.1	0.6	2.9

Table 3. Effect of IAA, IBA, NAA to induction roots of *Curcuma sp.*

PGR (mg/l)	Root induction rate (%)		Mean number root/culture		Mean length (cm)		Mean number bud/culture	
	after 10 d	after 30 d	after 10 d	after 30 d	after 10 d	after 30 d	after 10 d	after 30 d
IAA 0.5	70.0	100	1.3^{c}	2.8^{bc}	0.8^{de}	3.1^{c}	1.5^{c}	2.3^{b}
IAA 1.0	70.0	100	1.5^{b}	3.5^{b}	0.8^{de}	4.4^{ab}	2.0^{b}	3.3^{ab}
IAA 2.0	90.0	100	2.2^{a}	4.3^{a}	1.5^{a}	4.8^{a}	1.6^{bc}	2.3^{b}
IAA 3.0	73.3	100	1.6^{a}	3.2^{bc}	1.2^{b}	4.5^{ab}	1.5^{c}	2.2^{b}
IBA 0.5	76.7	100	1.6^{ab}	4.4^{a}	0.6^{d}	3.7^{cd}	2.8^{a}	3.0^{ab}
IBA 1.0	66.7	100	1.7^{ab}	4.8^{a}	0.6^{d}	4.0^{bc}	2.6^{a}	4.1^{a}
IBA 2.0	50.0	100	0.9^{c}	3.3^{bc}	1.0^{bc}	3.5^{cd}	1.9^{b}	3.4^{ab}
IBA 3.0	43.3	100	0.7^{c}	3.0^{c}	0.7^{cd}	2.9^{cd}	1.3^{c}	3.0^{ab}
NAA 0.5	26.7	100	0.4^{d}	2.5^{d}	0.2^{e}	1.8^{e}	1.8^{bc}	3.3^{ab}
NAA 1.0	46.7	100	0.8^{c}	2.6^{d}	0.3^{e}	2.2^{e}	1.8^{bc}	3.3^{ab}
NAA 2.0	60.0	100	1.5^{b}	3.5^{b}	0.4^{de}	2.8^{de}	1.5^{c}	2.5^{b}
NAA 3.0	40.0	100	0.8^{def}	1.9^{d}	0.2^{e}	2.6^{de}	1.2^{c}	2.0^{b}
CV%			1.2	0.8	0.8	0.4	0.7	0.8
$LSD_{0.05}$			0.7	1.2	0.3	0.6	0.6	1.2

PGR: Plant Growth Regulator.

recipes have the roots/bud in about 2 to 5 roots; this is not a significant number. The highest number of roots still belongs to IAA 2 mg/l and IBA 0.5 mg/l (4.3 - 4.4 root/bud), and all are the healthy roots, large diameter Figure 1(e) and Figure 1(f).

The highest length of roots belong to IAA recipes, the highest is IAA 2.0 mg/l recipe; IAA and IBA have the good effects for the length of roots in the *Curcuma sp.*'s tissue culture.

The results showed that all 12 recipes have created buds from 2 to 5 buds/samples; recipes of IBA have the highest number, especially IBA 1.0 mg/l has made 4.1 buds/sample after 30 days.

The difference between recipes of 4 indicators has the statistical confidence.

Based on the above results, we determined the optimal medium for *Curcuma sp.*'s root formation with formula MS's macronutrients ¼ + MS's micronutrients + Morel's vitamine + sucrose 25 g/l + coconut milk 10% + humate 2 ml/l + activated carbon 2 g/l + IAA 2.0 mg/l.

3.4. Medium for Multiple Shoots Formation

Curcuma sp. after rooting culture and bud sprouts, we transtered them to the medium multiple shoots formation; moved sample have 5 mm diameter including sprout, roots, each of which may have 1 to 2 sprout. Medium use the same ingredients as TA3 except macronutritrients MS 1/2, micronutrients MS, Morel's vitamine, 15% coconut milk, humate 3 g/l, additional phytohormone as Table 4.

Results in Table 4 shows the both kinds of cytokinin (kinetin and benzylaminopurin—

Table 4. Effects of cytokinin to bus of *Curcuma sp.* after 3 weeks.

PGR (mg/l)	Shoot induction rate (%)	No. of bud/culture after 3 weeks	Multiplier bud coefficient/week	Mean length shoot (cm)	No. of leaves per shoot
Kinetin 1.0	100	3.9[a]	1.1	3.5	2.0
Kinetin 2.0	100	4.0[b]	1.1	3.8	2.0
Kinetin 4.0	100	3.5[b]	1.0	4.0	2.4
Kinetin 6.0	100	3.1[c]	0.9	4.2	2.4
BAP 1.0	100	2.6[c]	0.7	4.3	2.0
BAP 2.0	100	5.7[a]	1.6	3.6	2.0
BAP 4.0	100	4.5[a]	1.3	3.5	2.4
BAP 6.0	100	2.8[c]	0.7	4.5	2.6
CV%		8.3	5.4	10.2	4.5
$LSD_{0.05}$	ns	1.3	0.6	1.4	0.8

BAP) are capable of producing buds (100%) at a concentration of 1.0 to 6.0 mg/l but different in multiplier bud coefficient, high of bud and number of leaves/bud. If the target is multiplier bud coefficient/week as high as possible, the BAP is better; the highest coefficient at BAP 2mg/l reaches 1.6 buds/week; kinetin only gets 1.0 buds/week. The height of buds and number of leaves/buds after 3 weeks there is the differences, but there is no statistical significance.

In experiment of medium selection for multiplier buds, TA3 medium additional BAP 2 mg/l has selected, giving the highest multiplier buds: 1.6 buds/week and the height of bud, number of leaves are qualified in tissue culture standards. This medium is containing to use for *Curcuma sp*'s propagation in a large scale and build plantlet *in vitro*.

3.5. Plantlet Regeneration

In the previous experiment, *Curcuma sp.* in tissue culture have become plantlet with roots (2 - 3 roots), 3 - 4 cm high, the number of leaves 2 - 3; although it is still in small size, weak to live *ex vitro*, so there must have provision steps: training *in vitro* plantlets, enough size (height, enough number of leaves, enough number of roots). The medium:

- Macronutrients: MS ½
- Micromutrients: MS
- Vitamine: Morel
- Humate: 3 g
- Coconut milk: 150 ml
- Activated carbon: 3g
- Composition phytohormone: IAA 2 mg/l + BAP 2 mg/l + TDZ 0.5 mg/l
- Agar: 7.5 g
- pH: 5.5 to 6.0

Samples being placed into tissue culture are controlled the number of plantlets in the 10 cm diameter bottle should have only 5 - 6 plantlets, brightness: 2000 lux, hours of light 12 h/day, temperature 25˚C - 260˚C.

Results after 4 weeks as follows:

Results of Table 5 show the culture medium for *Curcuma sp.* prior to *ex vitro* is appropriate, 5 criteria: the height of plantlet, the number of leaves/stem, the number of

Table5. Morphologic plantlet of *Curcuma sp*. after 4 weeks.

Mean height (cm)	No. of leaves/stem	No. of stems	No. of roots/stem	Mean diameter of stem (mm)
10.8 ± 2.1	4.1 ± 0.6	2.5 ± 0.3	4.1 ± 0.6	3.5 ± 0.3

stem, the number of roots and stem diameter are reached to the required level **Figure 1(h)**. After 6 weeks the plantlets are successfully transferred to *ex vitro* into net house **Figure 1(i)**.

4. Conclusion

Curcuma sp. is a rare medicinal species which is needed to be conserved and cultivated. Research results have shown that it is possible to propagate *Curcuma sp*. by tissue culture with MS's medium reinforcement by humate 2.0 - 3.0 g/l; *in vitro*, propagation coefficient reaches 1.6 buds/week, and after 6 months they can be produced plantlets to *ex vitro.*

Acknowledgements

This work was supported by grant from the Ministry of Education and Training of Vietnam. We thank Plant Tissue Culture Laboratory of Agriculture and Forestry Faculty, Tay Nguyen University.

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