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# Effects of Medium Salt Strength and Plant Growth Regulators on Shoot Multiplication and Root Induction of *Smilax corbularia*

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## Summary

Rhizomes of *Smilax corbularia* are used for the treatment of cancers, AIDS, septicemia and lymphatic diseases. The demand for rhizomes as an important ingredient in medicinal preparations has increased considerably. As rapid multiplication through *in vitro* propagation need to be developed, the effects of MS (Murashige and Skoog) medium salt strengths and plant growth regulators were investigated for shoot multiplication and root induction. For shoot multiplication, *in vitro* single-node explants were cultured on half strength MS (½ MS) or full strength MS (MS) media supplemented with 15% coconut water (cw) in combination with either 0.5-1.0 mg $l^{-1}$ BA (Benzyladenine) and 0-1.0 mg $l^{-1}$ IAA (Indole-3-acetic acid), or 0.5-2.0 mg $l^{-1}$ kinetin for six weeks. The results suggested that ½ MS medium supplemented with 15% cw and 1 mg $l^{-1}$ BA, and MS medium supplemented with 15% cw, 1 mg $l^{-1}$ BA combination with or without 0.5-1.0 mg $l^{-1}$ IAA gave the best shoot formations of 95-100%. The number of shoots and number of nodes per shoot obtained from these media were 1.0-1.3 and 4.4-5.0, respectively. For rooting, shoots were cultured on quarter strength MS (¼ MS), ½ MS or MS medium supplemented with 0-2 mg $l^{-1}$ NAA (α- Naphthalene acetic acid) for 12 weeks. The highest rooting percentages occurred on ¼ MS medium supplemented with 2.0 mg $l^{-1}$ NAA (65.83%) and ½ MS medium supplemented with 0.5 mg $l^{-1}$ NAA (66.67%), with the number of roots per shoot of 9.6 and 8.4, respectively. The rooted shoots were successfully transplanted with the survival rate of 80.81% in plastic pots containing soil, carbonized rice hull, decomposed rain tree leave, manure and sand at the ratio of 0.5:0.5:0.5:1:1.

KEYWORDS: MEDICINAL PLANTS, MICROPROPAGATION, ROOT INDUCTION, SHOOT MULTIPLICATION, SMILAX CORBULARIA

## Introduction

*Smilax corbularia*, a Thai medicinal plant, is known as Hua-Khao-Yen-Nua in Thailand (Koyama 1981, cited by 1). The rhizomes of this plant have been commonly used as an anti-inflammatory treatment in Thai traditional medicine. Two important compounds isolated from the rhizomes are astilbin and quercetin, which exhibit free radical-scavenging activity similar to that of L-ascorbic acid and Trolox. In addition, the plant has potential of anti-inflammatory property (2).

The rhizomes of *S. corbularia* have also been significantly used with other four species of Hua-Khao-Yen which are *Dioscorea birmanica*, *Dioscorea membranacea*, *Smilax glabra* and *Pygmaeopremna herbacea* for the treatment of cancers, AIDS, septicemia and lymphatic diseases (3). The demand for *S. corbularia* has increased creating the risk of uncontrolled harvesting and drastic reduction of plant populations in the forests of north and northeastern Thailand.

While sexual and vegetative propagation (rhizome cutting) methods are also possible, multiplication rates obtained from these methods remain low due to seed or rhizome dormancy, and uneven germination of rhizome. *In vitro* propagation is a promising solution to this problem as large number of desirable plant genotypes can be produced in a relatively short time.

Recently, a large number of endangered medicinal plants have been propagated using the plant tissue culture technique (4, 5, 6, 7). Micropropagation of other plant species of *Smilax* has been reported in *S. oldhami* (8, 9), *S. zeylanica* (10, 11), *S. glabra* (12) and *S. discotis* (13). However, similar approaches with respect to *S. corbularia* have yet to be fully described.

The objective of the present study was to investigate the effects of MS medium salt strength and exogenous plant growth regulators on the shoot multiplication and root induction of *S. corbularia* through the plant tissue culture technique.

## Materials and Methods

Plant material and nodal preparation

Rhizomes of *S. corbularia* (Fig. 1a) were collected from native forest habitats in Chiang Mai, Thailand (18° 47' 43" N/ 98° 59' 55" E) and transplanted in the greenhouse at Thammasat University, Pathumthani, Thailand (14 ° 1' 0" N/100° 32' 0" E). After 5-6 weeks of germination, aerial stems (Fig. 1b) were collected and cut into pieces approximately 1 cm length, each with a single lateral bud. Explants were thoroughly washed with tap water, the surface sterilized with 10% and 5% (v/v) sodium hypochlorite solution for 25 and 20 min, respectively and finally rinsed twice in sterile distilled water. The nodal segments were inoculated vertically into MS medium supplemented with 1 mg/l BA supplemented with 15% coconut water (cw) for shoot induction.

### Media and culture conditions

MS (Murashige and Skoog) medium was added with sucrose at the concentration of 3% before adjusting pH to 5.8 with 1N NaOH. The medium was gelled with 8% agar and dispensed into glass vessels prior to autoclaving at 121°C for 15 min. All cultures were maintained at 25±2°C under a 16 h photoperiod provided by white fluorescence tubes with an intensity of 3,000 lux.

### Shoot multiplication

From the preliminary experiment, no shoot induction was observed on a hormone-free MS medium while BA promoted shoot formation but regenerated shoots were short. Higher percentage of shoot formation and healthy shoots occurred on MS medium supplemented with BA and 15% cw. Therefore, the effects of MS medium salt strength and concentrations of cytokinin and auxin on shoot multiplication were investigated in this experiment. The *in vitro* grown shoots after 2 months of culture were used as the starting material. The shoots were cut into segments, each containing one lateral bud (about 1 cm length) and cultured on 16 different

media: ½ MS or MS medium supplemented with 15% cw, in combination with either 0.5-1.0 mg $l^{-1}$ BA and 0-1.0 mg $l^{-1}$ IAA, or 0.5-2.0 mg $l^{-1}$ kinetin. A complete randomized design (CRD) with 10 replicates was used. Each replicate had two vessels with 1 nodal explant in each. The percentage of shoot formation, number of shoots and number of nodes per shoot were recorded after culturing for 6 weeks.

### Root induction and transplantation

*In vitro* shoots of about 2 cm in length were cultured on ¼ MS, ½ MS or MS media supplemented with 0, 0.5, 1.0 and 2.0 mg $l^{-1}$ NAA. The experiment was arranged in CRD with 12 treatments and 10 replicates. Each replicate had 3 vessels with 1 shoots in each. Rooting percentage, number of roots per shoot, root length and time required for rooting were recorded 12 weeks later.

The rooted shoots (2-3 cm in height, 3-4 leaves and 6-8 roots) were washed in water and transferred to 17.5 x 25 cm plastic boxes containing moist cocopeat (forty shoots per box) and covered with the plastic lids to maintain high humidity. The boxes were kept in the greenhouse at 30-35°C with natural light and the lids were opened after 2 weeks. The acclimatized plants were transferred to plastic pots (12.7 cm diameter) containing soil, carbonized rice hull, decomposed rain tree leave, manure and sand at the ratio of 0.5:0.5:0.5:1:1. Survival rate was recorded after transplantation in the plastic pots for 4 weeks.

### Statistical analysis

Analysis of variance (ANOVA) was used to determine the statistical significance of the difference between treatment means in all experiments. ANOVAs were calculated using SAS version 9.1 for Microsoft Window®. Where a significant difference was found, the Duncan's New Multiple Range Test (DMRT) at the 5% level of probability was used to compare individual treatment means.

## Results and Discussion

### Shoot multiplication

After four weeks of culture, there were significant differences among the treatments in percentage of shoot formation, number of shoots and number of nodes per shoot (Table 1). The percentages of shoot formation on ½ MS or MS media supplemented with 15% cw, 0.5 -1 mg $l^{-1}$ BA and 0-1 mg $l^{-1}$ IAA were in the range 85-100%, while between 60-85% of shoot formation occurred on ½ MS or MS medium supplemented with 15% cw and 0.5-2 mg $l^{-1}$ kinetin. The number of shoots (0.8-1.3) and node number per shoot (2.3-5.0) on ½ MS or MS medium supplemented with 15% cw, BA and IAA tended to be higher than those on ½ MS or MS medium supplemented with 15% cw and kinetin, as 0.7-0.9 and 0.9-1.6, respectively. These results indicated that the type of cytokinin significantly affected the percentage of shoot formation, shoot number and number of nodes per shoot, while varying MS medium salt strengths had no effect. Of the two cytokinins (BA and kinetin) tested, BA was more effective than kinetin when used singly or with the auxin IAA. BA is regarded as the most efficient cytokinin for promoting shoot multiplication in many plant genera, including *Smilax* such as *S. oldhami* (8), *S. zeylanica* (10), and *S. discotis* (13). However, other studies on *S. zeylanica* have reported that enhanced shoot multiplication was also obtained from differentiating calli (14) or nodal segments (11) upon the kinetin-containing medium. This suggests that exogenous hormone requirements depend upon endogenous levels within the plant system, which vary with tissue type, plant type and plant growth phase (15). A low number of shoots (0.7-1.3) were also observed in this plant species, possibly because *S. corbularia* is a woody plant (16). Linington (17) commented that, while the *in vitro* propagation technique has been highly successful with herbaceous species, most woody species respond poorly, possibly due to a low rate of shoot proliferation, poor growth and phenolic exudation. A similar problem appears to exist in *S. zeylanica*, in which only two shoots formed when

the nodal segments were cultured on ½ MS medium supplemented with 2 mg $l^{-1}$ kinetin, 50 mg $l^{-1}$ L-glutamine and 100 mg $ml^{-1}$ activated charcoal (11). The data presented in Table 1 suggest that the optimal media for shoot multiplication of *S. corbularia* were ½ MS medium supplemented with 15% cw and 1 mg $l^{-1}$ BA, and MS medium supplemented with 15% cw, 1 mg $l^{-1}$ BA combination with or without 0.5, 1.0 mg $l^{-1}$ IAA (Fig. 2a), since these media promoted the highest multiplication rates (shoot number x number of nodes per shoot).

**Root induction**

Root formation occurred on all rooting media within four weeks. Rooting percentages varied from 24.81% to 66.67%, with no significant difference among treatments (Table 2). The rooting percentage was higher than 65% when shoots were cultured on ¼ MS medium supplemented with 2 mg $l^{-1}$ NAA and ½ MS medium supplemented with 0.5 mg $l^{-1}$ NAA (Fig. 2b). The highest number of roots obtained was 9.6 on ¼ MS medium supplemented with 2 mg $l^{-1}$ NAA, which was not significantly different from those cultured on ¼ MS medium supplemented with 0.5 mg $l^{-1}$ NAA, ½ MS supplemented with 0.5 or 1.0 mg $l^{-1}$ NAA and MS medium supplemented with 1 or 2 mg $l^{-1}$ NAA. A significant difference in root length was found among the treatments. The ½ MS medium supplemented with 0.5 mg $l^{-1}$ NAA induced the longest roots of 1.24 cm while shoots grown on MS medium supplemented with 0 and 0.5 mg $l^{-1}$ NAA had the shortest roots of 0.17 and 0.15 cm, respectively. *S. corbularia* plantlets with expanded leaves and well developed roots were successfully acclimatized and transplanted in plastic boxes (Fig. 3a). The survival rate was 80.81% after 4 weeks of transferring the acclimatized plants to plastic pots containing soil and other growing substrates (Fig. 3b).

Even though the percentage of rooting and time required for *in vitro* rooting were not significantly different, it appears that ¼ MS and ½ MS media produced superior results to the full strength MS medium. This finding is in agreement with observations made of other plant species, including *Justicia gendarussa* (5), *S. zeylanica* (10), *S. discitis* (13), *S. zeylanica* (14), and *Dioscorea nipponica* (18). Bhojwani and Razdan (15) suggested that the nutrient salts in the medium had dramatic effects both on rooting percentage and root number. Generally, where shoot multiplication was induced on full strength MS medium, the salt concentration was reduced to half or a quarter for rooting. Wang (1978 cited in 11) also reported that reduction in salt concentration might promote the root development in many woody plants, a phenomenon probably associated with reduced nitrogen levels (15). The addition of plant growth regulators, especially auxins such as IBA and NAA, is required to induce rooting in woody plants (19).

The stimulation of rooting by NAA observed in this study agrees with similar findings involving other medicinal plant species of *Smilax,* including *S. oldhami* (9) and *S. discotis* (13). From the results, therefore, the optimal media for root induction of *S. corbularia* were ¼ MS supplemented with 2.0 mg $l^{-1}$ NAA and ½ MS supplemented with 0.5 mg $l^{-1}$ NAA.

In conclusion, types of plant growth regulator have a significant effect on shoot multiplication in *S. corbularia,* while root induction was affected either by MS medium salt strength or plant growth regulators. It is possible to produce *in vitro S. corbularia* plantlets, and it is expected that the protocols reported here for the first time will facilitate rapid multiplication of *S. corbularia*, a valuable medicinal plant.

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| MS salt strength | Concentration (mg $l^{-1}$) | | | Shoot formation (%) | Number of shoots[1/] | Number of nodes/shoot |
|---|---|---|---|---|---|---|
| | BA | IAA | kinetin | | | |
| ½ | 0.5 | 0.5 | - | $95^{ab}$ | $1.2^{ab}$ | $3.2^{bcd}$ |
| ½ | 0.5 | 1 | - | $90^{abc}$ | $1.0^{abcd}$ | $2.5^{de}$ |
| ½ | 1 | - | - | $95^{ab}$ | $1.1^{ab}$ | $5.0^{a}$ |
| ½ | 1 | 0.5 | - | $85^{abc}$ | $0.8^{bcd}$ | $3.0^{cd}$ |
| ½ | 1 | 1 | - | $95^{ab}$ | $1.1^{ab}$ | $2.3^{de}$ |
| ½ | - | - | 0.5 | $65^{bc}$ | $0.7^{cd}$ | $1.1^{f}$ |
| ½ | - | - | 1 | $65^{bc}$ | $0.7^{cd}$ | $1.3^{f}$ |
| ½ | - | - | 2 | $85^{abc}$ | $0.9^{abcd}$ | $1.6^{ef}$ |
| 1 | 0.5 | 0.5 | - | $90^{abc}$ | $0.9^{abcd}$ | $4.1^{abc}$ |
| 1 | 0.5 | 1 | - | $80^{abc}$ | $0.8^{bcd}$ | $3.3^{cd}$ |
| 1 | 1 | - | - | $95^{ab}$ | $1.2^{ab}$ | $4.5^{ab}$ |
| 1 | 1 | 0.5 | - | $100^{a}$ | $1.3^{a}$ | $4.4^{ab}$ |
| 1 | 1 | 1 | - | $100^{a}$ | $1.0^{abcd}$ | $4.7^{a}$ |
| 1 | - | - | 0.5 | $60^{c}$ | $0.7^{cd}$ | $0.9^{f}$ |
| 1 | - | - | 1 | $65^{bc}$ | $0.7^{cd}$ | $1.2^{f}$ |
| 1 | - | - | 2 | $65^{bc}$ | $0.7^{cd}$ | $1.2^{f}$ |
| F-test | | | | * | * | * |
| C.V. (%) | | | | 39.86 | 19.84 | 26.38 |

Table 1 Percentage shoot formation, number of shoots and number of nodes per shoot after single nodal segments were cultured on ½MS or MS medium supplemented with 15% cw and varying concentrations of BA, IAA or kinetin.

| MS salt strength | NAA (mg $l^{-1}$) | Rooting (%) | Number of roots per shoot[1/] | Root length (cm) | Days to rooting |
|---|---|---|---|---|---|
| ¼ | - | 63.33 | $3.3^{bcd}$ | $1.00^{ab}$ | 22.4 |
| ¼ | 0.5 | 55.83 | $6.5^{abc}$ | $0.94^{ab}$ | 19.4 |
| ¼ | 1 | 30.00 | $4.3^{bcd}$ | $0.62^{abc}$ | 9.8 |
| ¼ | 2 | 65.83 | $9.6^{a}$ | $0.75^{abc}$ | 26.0 |
| ½ | - | 43.33 | $3.2^{bcd}$ | $0.47^{bc}$ | 17.3 |
| ½ | 0.5 | 66.67 | $8.4^{ab}$ | $1.24^{a}$ | 24.3 |
| ½ | 1 | 26.67 | $5.8^{abcd}$ | $0.49^{bc}$ | 8.6 |
| ½ | 2 | 43.33 | $3.8^{bcd}$ | $0.57^{abc}$ | 19.1 |
| 1 | - | 24.81 | $0.4^{d\ 1/}$ | $0.17^{c}$ | 10.6 |
| 1 | 0.5 | 32.59 | $1.1^{cd}$ | $0.15^{c}$ | 12.9 |
| 1 | 1 | 41.11 | $5.0^{abcd}$ | $0.40^{bc}$ | 12.7 |
| 1 | 2 | 43.33 | $7.7^{ab}$ | $1.05^{abc}$ | 13.5 |
| F-test | | ns | * | * | ns |
| C.V. (%) | | 41.01 | 29.7 | 15.25 | 32.0 |

Table 2 Effect of different concentrations of MS salt strength and NAA on root induction of *Smilax corbularia*. Data recorded after 12 weeks of culturing.

[1/] Means followed by the same letter in a column are not significantly different at the 5% level of probability by DMRT.

ns = No significant difference at the 5% level of probability.

* = Significant difference at the 5% level of probability


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Fig. 1 *Smilax corbularia*. (a) Rhizome; (b) Aerial stem

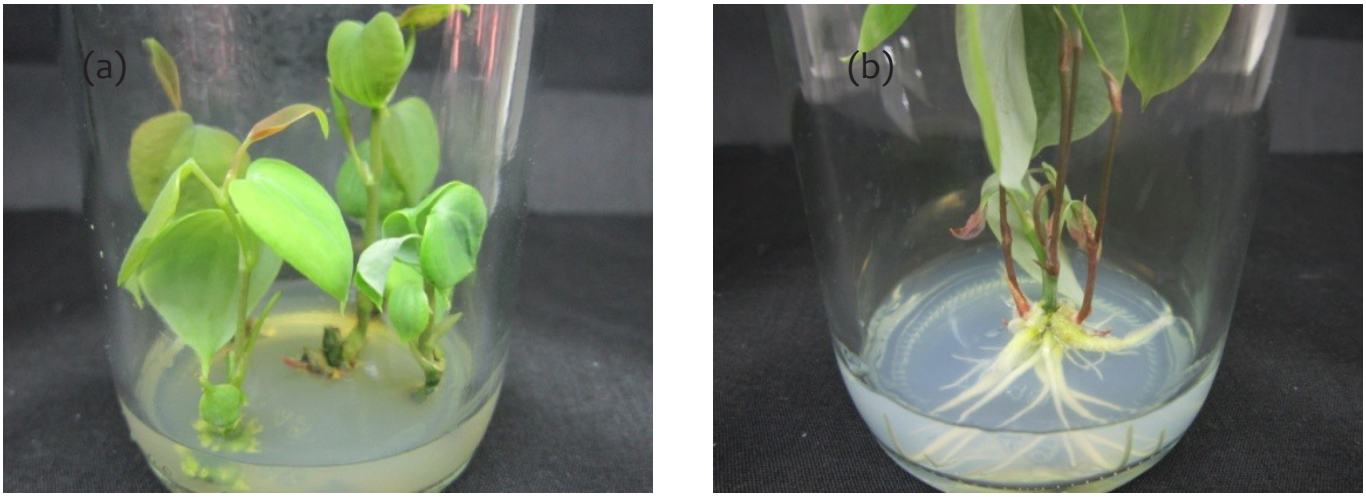


Fig. 2 *Smilax corbularia* shoot multiplication and rooting. (a) Regenerated shoots on MS medium supplemented with 15% cw, 1 mg $l^{-1}$ BA and 0.5 mg $l^{-1}$ IAA; (b) Rooted shoot on ½ MS supplemented with 0.5 mg $l^{-1}$ NAA

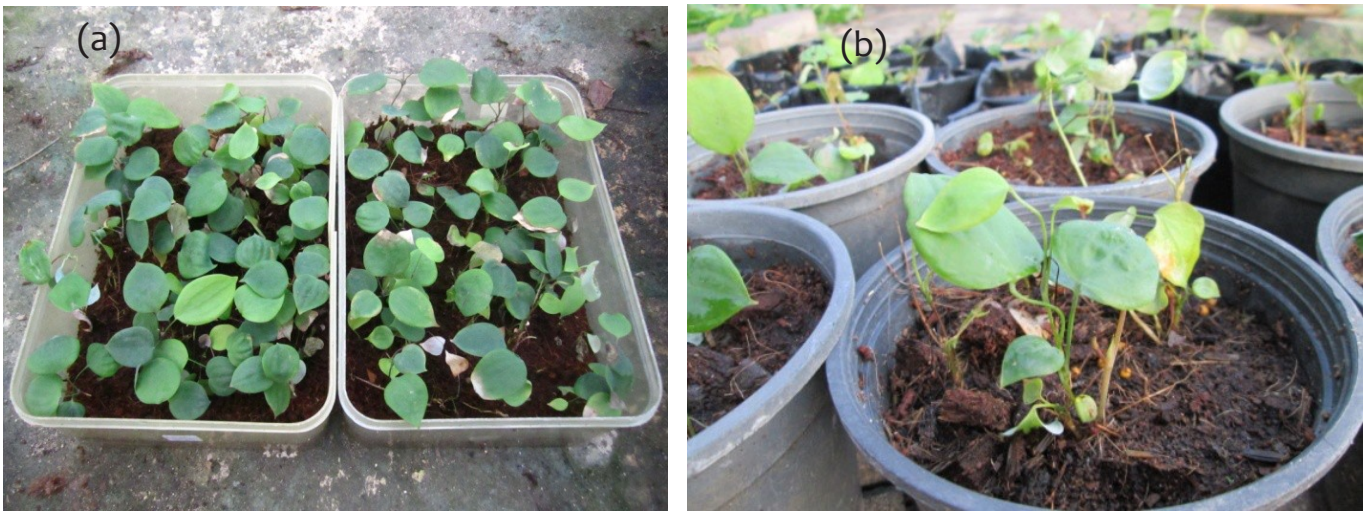


Fig. 3 (a) Acclimatized plantlets after 2 weeks of transplantation in plastic boxes containing moist cocopeat; (b) Healthy plants after transplantation in plastic pots