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A new method to increase plant quantity through tissue culture on *Vitex negundo* L.

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Abstract A new method to increase plant quantity through tissue culture on *Vitex negundo* L. was discovered. The influences of PGRs on shoot regeneration cultured on MS medium with 0.5, 1, 2, or 3 mg/L benzylaminopurine (BAP), meta-Topolin (*mT*) and Thidiazuron (TDZ) were resulted to be the greatest percentage of shoot development was 100% at 1 mg/L BAP in MS medium. The greatest average number of shoot multiplication was 4.40 shoots at 0.5 mg/L BAP in MS medium after 8 weeks of culture. For roots, the result of MS media fortified with 0.5, 1, 2, and 3 mg/L of indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) discovered that the excellent percentage of root production was 90% at 3 mg/L IBA. The highest average number of root production was 108.20 roots on 2 mg/L NAA after 8 weeks of culture. The plantlets were eventually transferred into the plastic pots. In this research work, the nodes were demonstrated to be an outstanding regenerating explant resource for *Vitex negundo* L. An effective regeneration strategy for plantlets derived from the nodes was demonstrated.

Keywords: *Vitex negundo* L., node, root induction, shoot regeneration, plant growth regulators

Introduction

Vitex negundo L., or Chinese chaste tree is a woody plant, or shrub, belonging to the Verbenaceae family. It is one kind of essential herbal plant (Reddy *et al*., 2014); and is considered a local plant of East Asia, South West China, throughout India in warmer zones and the western Himalayas; and is also cultivated in Pakistan (Khan *et al*., 2007; Khare, 2007). *Vitex* spp. is also well known in Thailand for its 4 cultivars: *Vitex trifolia* Linn., *Vitex trifolia* var. purpurea, *Vitex trifolia* subsp. litoralis Steenis or *Vitex rotundifolia* L.f., and *Vitex negundo* L. (BGO Plant Databases, The Botanical Garden Organization, 2018), which are popularly helpful due to their high nutrients composition and

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suitable for medicinal use. Specifically, *Vitex trifolia* Linn. is one of the ingredients for brain stimulation, fever reduction, pain relief, and hematinic. Moreover, the extracts from various parts of the plants can control any bacterial activity, provide hair nourishment and scalp care (as anti-dandruff shampoo), has anti-inflammation properties, and diabetes- and cancer-preventing abilities (Abdulrahman *et al.*, 2017). The plant has strong mosquito repellent action (Nguyen-Pouplin *et al.*, 2007), and against Aedes aegypti (Hebbalkar *et al.*, 1992). It also shows antibacterial activity against *Escherichia coli, Klebsiella aerogenes, Proteus vulgaris and Pseudomonas aerogenes* (Samy *et al.*, 1998). However, the International Union for Conservation of Nature (IUCN) reported on the extinction risk of *Vitex* spp., due to its low growth rate. These herbal plants have been unrestrictedly overexploited for the preparation of numerous beneficial medicines leading to a dramatic reduction in their growth (Nagaveni and Rajanna, 2013). Therefore, one of the most important goal was to protect *Vitex* spp. from extinction. The research finding aimed to develop a new method to increase plant quantity through tissue culture on *Vitex negundo* L.

Materials and methods

Sterilization and effect of plant growth regulators on shoot regeneration

The nodes of Chinese chaste (*Vitex negundo* L.) were washed through water for 10 minutes. The nodes were sterilized with 0.1% Mercuric (II) Chloride ($HgCl_2$) and 2 drops of Tween 20 in 80 mL, followed by shaking at 250 rpm for around 4 minutes. Then, the nodes were transferred to sterilized water and shaken for around a minute 4 times. After that, the nodes were moved to treat on MS medium (Murashige and Skoog, 1962) fortified with 0.5, 1, 2, or 3 mg/L of 6-Benzylaminopurine (BAP), *meta*-Topolin (*m*T), Thidiazuron (TDZ) and without PGRs, 30 g/l sucrose and 2.6 g/l phytagel. The growths were observed and recorded by enumerating the number of nodes germination and measuring the shoot length using a Vernier caliper after 8 weeks of culture.

Rooting

After the experiment of shoot regeneration, the shoots were transferred to MS medium supplemented with 0.5, 1, 2, or 3 mg/L of Indole-3-Butyric Acid (IBA) and Naphthalene acetic acid (NAA). The roots and shoot regeneration were observed and recorded by enumerating the number of root germination and measuring the shoot length using a Vernier caliper after 8 weeks of culture.

Before sterilization, the pH of the medium was 5.8, and it was sterilized at 121℃ for 15 minutes; it was fortified with 30g/L sucrose and 0.26 g/L phytagel. At 25±2°C, the experiments were incubated for 16 hours photoperiod.

Plant acclimatization

The plantlets were transplanted into sterilized soil in plastic pots. They were sealed with plastic bags to retain humidity for 4 weeks. After that, the plantlets were potted and grown in the greenhouse.

Data analysis

In each medium, there were ten explants. The data were analyzed using a one-way analysis of variance, and significant differences between means were determined using Duncan's multiple range test at a probability of 5% ($p>0.05$) in SPSS (Statistics Package for the Social Sciences) version 23. (IBM SPSS Statistics 23).

Result

Effect of plant growth regulators on multiplication of shoots

The influence of PGRs on shoot regeneration cultured on MS medium with 0.5, 1, 2, or 3 mg/L BAP, *m*T, and TDZ resulted in the following. The node explants cultured for 8 weeks showed the highest shoot regeneration rate at 100% in MS medium combined with 1 mg/L BAP; whereas the highest average number of shoots was at 0.5 mg/L BAP. The greatest average of shoots and shoot length at 0.5 mg/L *m*T were 4.40 shoots/explant at 0.5 mg/L BAP and 2.03 cm/shoot, respectively, better than other concentrations. Node explants cultured on 3 mg/L *m*T had the lowest shoot regeneration rate at 20% (Table 1). The experiment results indicated that BAP and TDZ induced shoots better than *m*T. Shoot regeneration cultured on MS medium combined with 0.5 mg/L BAP had the most average number and length of shoots, which were 4.40 shoots/explant and 1.83 cm/shoot, respectively. The characteristic of shoots cultured in MS medium fortified with BAP showed the highest plant growth in green and about 4-5 shoots and leaves (Figure 1B). At a high concentration of BAP, shoots responded with hypersensitivity. The MS medium plus 1 mg/L *m*T had the best average number and shoot length: 3.20 shoots/explant and 1.45 cm/shoot, respectively; the shoot was characterized by short green shrubs

(Figure 1C). The lower concentrations of *m*T had a greater numeral of shoots than those in higher concentrations.

Meanwhile, the MS medium combined with 0.5 mg/L TDZ had the best average number and shoot length: 3.60 shoots/explant and 0.99 cm/shoot, respectively. Shoots were short, green, and callus on the base (Figure 1D). The characteristics of shoots in MS medium without PGRs were long, green in color, and about 2-3 shoots and leaves (Figure 1A), similar to MS medium supplemented with BAP (Figure 1B).

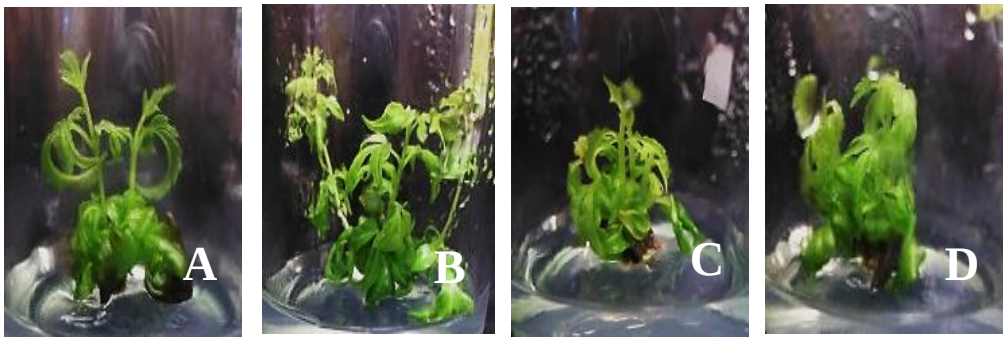

Figure 1. Proliferation characteristic of Chinese chaste tree shoots in MS medium with various concentrations of PGRs after being cultured for 8 weeks: (A) shoots induced on MS medium without PGRs;(B) 0.5 mg/L BAP; (C) 1 mg/L *m*T.; (D) 0.5 mg/L TDZ

Table 1. Effect of the various BAP, mT, and TDZ concentrations in MS medium for shoot regeneration from nodes of Chinese chaste tree after being cultured for 8 weeks

PGRs (mg/L)		**Number of shoot regeneration(%)**	**Average number of shoots**	**Average length of shoots (cm)/1,2,3**
0 (Control)		8 (80)	2.00 ±0.00	2.04 ±0.51a
BAP	0.5	8 (80)	4.40 ±0.54	1.83 ±0.10a
	1	10 (100)	3.00 ±1.00	0.99 ±0.07cde
	2	6 (60)	3.00 ±1.00	1.02 ±0.17cd
	3	8 (80)	3.60 ±0.54	1.18 ±0.02bc
mT	0.5	9 (90)	2.00 ±0.00	2.03 ±0.59a
	1	8 (80)	3.20 ±1.09	1.45 ±0.29b
	2	8 (80)	2.20 ±0.44	0.97 ±0.22cdef
	3	2 (20)	2.00 ±0.00	0.63 ±0.05efg
TDZ	0.5	7 (70)	3.60 ±1.51	0.99 ±0.09cde
	1	4 (40)	3.40 ±0.89	0.56 ±0.05g
	2	4 (40)	3.20 ±1.64	0.61 ±0.06fg
	3	6 (60)	2.00 ±0.00	0.76 ±0.27defg

[1]/ Ten explants in each media.
[2]/ Each value represents the mean +- SD of 10 explants.
[3]/The data were statistically analysis using Duncan's multiple range test (DMRT). In the same column, significant differences at $P \leq 0.05$ level are indicated by different letters

Rooting

Shoot transferred from the previous experiment to MS medium supplemented with 0.5, 1, 2, or 3 mg/L of IBA and NAA for root induction. It resulted to the following after 8-week culture, the highest root regeneration rate, average number of roots, and average length of root were 90% at 3 mg/L IBA, 108.20 roots/explant at 2 mg/L NAA, and 3.15 cm/shoot, respectively (Table 2). Root induction cultured on MS medium combined with IBA found that 1 mg/L IBA had the maximum average number of roots and root length were 93.00 roots/shoot and 3.15 cm/root, respectively. The characteristic of the root was yellowish, white a more significant number of roots, thin size and elongated (Figure 2B). MS medium combined with NAA revealed that the 2 mg/L NAA had the uppermost average root number and length of the root which were 108.20 roots/shoot and 2.73 cm/root, respectively. Root was characterized as yellowish white and had more numbers. Although at 2 mg/L NAA, roots were characterized to be long and thin (Figure 2C), another concentration had a short length and stunt. The result showed that IBA had better root induction than NAA; whereas NAA induced more root. Moreover, the result indicated that the lower concentrations of IBA and NAA had more generated roots than the higher concentration. MS medium without PGRs had the lowest average number of roots (3.60 roots/shoot); roots were yellowish white, thin in size, and short in length (Figure 2A).

For plant acclimatization, the plantlets were transplanted into sterilized soil in plastic cups. The result found that the growth was successful inside a greenhouse after 4-week culture (Figure 3A).

Table 2. Effect of various IBA and NAA concentrations in MS medium for root regeneration of Chinese chaste tree after being cultured for 8 weeks

PGRs (mg/L)		Number of root regeneration (%)	Average number of roots	Average length of root (cm)/1,2,3
Control		4 (40)	3.60 ±2.19	1.64 ±0.41de
IBA	0.5	6 (60)	68.00 ±28.84	2.72 ±0.04b
	1	3 (30)	93.00 ±13.98	3.15 ±0.03a
	2	7 (70)	41.20 ±13.88	1.81 ±0.03cd
	3	9 (90)	24.60 ±3.58	1.33 ±0.01f
NAA	0.5	2 (20)	61.80 ±25.04	1.74 ±0.11de
	1	3 (30)	94.20 ±38.32	1.97 ±0.10c
	2	5 (50)	108.20 ±55.73	2.74 ±0.22b
	3	4 (40)	51.40 ±24.29	1.54 ±0.07ef

[1]/ Ten explants in each media.
[2]/ Each value represents the mean +- SD of 10 explants.
[3]/The data were statistically analysis using Duncan's multiple range test (DMRT). In the same column, significant differences at $P \leq 0.05$ level are indicated by different letters

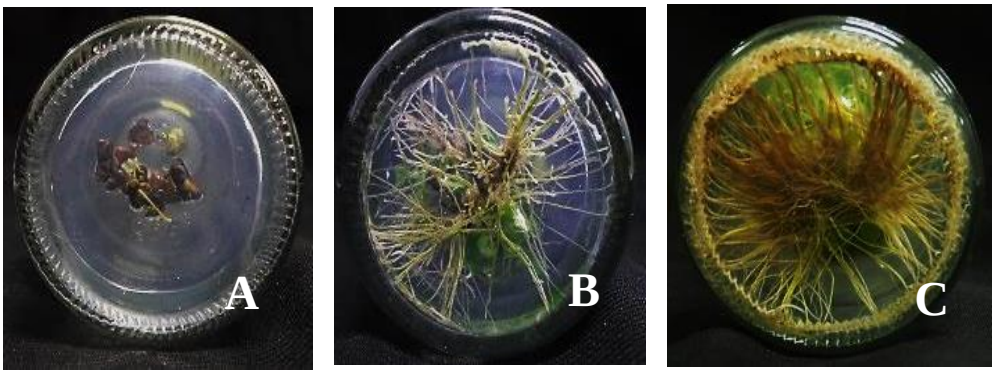

Figure 2. Characteristics of root induction of Chinese chaste tree cultured on MS medium fortified with different concentrations of PGRs after 8 weeks of culture: (A) rooting on MS medium without PGRs; (B) root emerging on MS medium combined with 1 mg/L IBA; and (C) root induction on MS medium combined with 2 mg/L NAA

Figure 3. (A) Observed characteristics of plant acclimatization of Chinese chaste tree after 4 weeks

Discussion

Chinese chaste tree is widely used in South West China, throughout India in warmer zones and Western Himalayas, and cultivated in Pakistan. It has numerous health benefits, such as relieving pain, reducing fever, controlling or inhibiting bacterial growth, and preventing diabetes and cancer. Consequently, there is a need to study, develop, and improve the plant culture method in vitro

through an efficient method for propagating the seedlings of plants and providing enough quantities to meet the demand. This study found that lower concentrations of all PGRs, BAP, and TDZ had a higher number of shoots than higher concentrations, which could induce shoots better than *m*T. Inconsistent with Dar *et al*. (2012), who reported that culturing of *Vitex negundo* on MS medium with 0.5 mg/L BAP had 80% shoot regeneration, and the average number of shoots was 2 shoots/explant after 40-day culture. This is similar to Ngoenngam *et al*. (2015) that tested *Vitex trifolia* var. purpurea and *Vitex trifolia* subsp. litoralis Steenis cultured on MS medium with various concentrations of BAP found that the best result of shoot generated at 0.5 mg/L BAP (1.67 shoots/explant) and 1 mg/L BAP (4.33 shoots/explant). Nagaveni and Rajanna (2013) correspondingly reported that *Vitex trifolia* cultured on MS medium plus 9.84 µM IBA had the highest number of roots which was 11-14 roots/shoot after being cultured for 30-40 days, while MS medium without PGRs had no root in their study. Reddy *et al*. (2014) observed that root induction of Chinese chaste tree on MS medium combined with 0.5 mg/L IBA had 87% root induction rate (6.4 roots/shoot) after being cultured for 3-4 weeks, and MS medium with NAA concentration of 0.5 had no root generation.

Ultimately, the plantlets were successfully transplanted into the plastic pots. The nodes were proven to be an excellent regenerating explant resource of the Chinese chaste tree in this research study. An effective regeneration strategy for plantlets originating from the nodes was confirmed.

Acknowledgments

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