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Full Length Research Paper

Comparative study on different methods for *Lonicera japonica* Thunb. micropropagation and acclimatization

Jiang Xiang Hui[1,2], She Chao Wen[2], Zhu Yong Hua[1] and Liu Xuan Ming[1]*

[1]Bioenergy and Biomaterial Research Center, College of Biology; State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan University, Changsha 410082, Hunan, China.
[2]Department of Life Science, Huaihua University, Huaihua, Hunan 418008, China.

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In this study, we reported the establishment of a simple protocol for the micropropagation and acclimatization of *Lonicera japonica* Thunb. Branches with dormant buds were collected from mature *L. japonica* and sprouted in a greenhouse. Tip and node segments were used as starting material for *in vitro* proliferation in woody plant medium (WPM). In the first assay in which explants from five different species of *Lonicera* were used, 95.0% of the tip segments produced new axillary shoots, thus proving to be the best explant type. Afterwards, material from *L. japonica* Thunb. was used to test for plant growth regulator (PGR) combination. Shoots from *L. japonica* were used to assay *in vitro* rooting using six different WPM media. Rooting percentages were high for all media and varied between 83 and 95%. For acclimatization, two approaches were assayed: the use of previously rooted *in vitro* plants and the direct acclimatization of shoots. After five weeks, 95.0% of the *in vitro* rooted plants were successfully acclimatized and 88.7%, was attained by direct acclimatization of shoots with two years shoot immersed in GA_3 solution. These results proved that there is no need for a previous *in vitro* rooting step and that direct acclimatization can effectively reduce time and costs. Thus, the micropropagation and acclimatization of *L. japonica* can be divided into only two steps: proliferation of shoots in WPM and direct acclimatization of these shoots in a sterile soil mixture, or direct acclimatization of two years shoot immersed in GA_3 solution for 8 h.

Key words: *Lonicera japonica* Thunb., node segment culture, micropropagation, direct acclimatization.

INTRODUCTION

Lonicera japonica Thunb. is a genus of woody plants (family Caprifoliaceae) that grows extensively in Europe, Asia and North America. *L. japonica* Thunb. is recorded in China Pharmacopeia (2005) with the Chinese name Jinyinhua. The aqueous extract from *L. japonica* Thunb. flower has been used in Chinese traditional medicine for treating fever, arthritis and infectious diseases for thousands of years. This plant has been shown to display a wide spectrum of biological and pharmacological activities such as antibacterial, antiviral (Houghton et al., 1993), antioxidant and inhibition of the platelet activating factor (Kim et al., 1994). *L. japonica* could act as an anti-inflammatory agent through regulation of NF-κB activation (Lee et al., 2001). Rutin is one of the key compounds of *L. japonica*; rutin has been shown to provide protection against ischemia and reperfusion (I/R) in a variety of experimental models and via multiple mechanisms (Lanteri et al., 2007; Lao et al., 2005). *L. japonica* contains anti-complementary polysaccharides and polyphenolic compound. The polyphenolic compounds inhibit the platelet aggregation, thromboxane biosynthesis and hydrogen peroxide induced endothelial injury (Chang

*Corresponding author. E-mail: xmll05@126.com.

Abbreviations: ANOVA, Analysis of variance; **BA,** N_6-benzylaminopurine; **IAA,** indole-3-acetic acid; **IBA,** butyric acid; **NAA,** α-naphthaleneacetic acid; **WPM,** woody plant medium; **PGR,** plant growth regulator.

and Hsu, 1992). This species is rich in iridoid secologanin (Son et al., 1994) and is a potentially useful model for the study of secologanin biosynthesis. Secologanin is a primary terpenoid intermediate in the biosynthesis of monoterpenoid indole alkaloids such as reserpine, ajmaline, ajmalicine and vinbiastine (Zenk, 1980).

L. japonica Thunb. seeds have the problem of low germination rate, low orderliness and long seedling time. The rapidness of tissue culture techniques can be advantageous for the continuous provision of a plantlet stock for cultivation and may further compliment breeding programmes. The strategies for *L. japonica* production involved somatic embryogenesis and direct acclimatization of shoots. Georges et al. (1993) proved that shoot regeneration from true-callus (without any part of the original explant) was achieved for the three different source tissues within 12 weeks, which were morphologically and genetically similar to the mother plant. Palacios et al. (2002) studied the regeneration of *Lonicera tatarica* plants from cultured stem sections, which showed that the age of the donor plant had no noticeable effect on either process; however, rooting of elongated shoots occurred only with shoots derived from 2-month-old donor plants, the plants regenerated from stem explants were morphologically normal and levels of loganin and secologanin were comparable to those detected in plants grown from seed. Cambecedes et al. (1991) investigated the effect of growth regulators on bud regeneration from leaf explants of the shrubby ornamental honeysuckle genotype *Lonicera nitida*, which showed that caulogenesis cannot be managed only by the classical modification of the exogenous cytokinin/ auxin balance, and the right hormonal balance to achieve such a goal must also involved a control of the endogenous auxin level of explants.

In view of the demand of amplification culture and the drastic reduction in the number of excellent genus, micropropagation offers many advantages because it potentially can facilitate large-scale production of valuable genus and allow plant regeneration from genetically modified cultures. In addition, micropropagation may be an essential step to obtain plants from frozen collections of *L. japonica* Thunb. Unfortunately, despite the considerable number of micropropagation reports aforementioned, most of the protocols are poorly described, in particular concerning the difficult stage of acclimatization. Therefore, our goal was to try to develop a reliable and comprehensive protocol for *L. japonica* Thunb. micropropagation and acclimatization.

MATERIALS AND METHODS

In previous experiments, *in vitro* culture of *L. japonica* Thunb. shoots collected in the field during spring resulted in a contamination rate of 85 to 90%. Collection of branches with dormant buds reduced culture contamination to 0 to 10% and was thereafter the chosen method to obtain starting material for cultures.

Branches with dormant buds were collected from five different species of *Lonicera* in January to March, such as *L. japonica* Thunb., *L. confusa* (Sweet) DC., *L. dasystyla* Rehd., *Lonicera hypoglauca* Miq. and *Lonicera macranthoides* Hand. -Mazz., located at Huaihua University medicinal garden.

The first assay of shoot proliferation was performed with material from these five different species of *Lonicera* to determine which explant behaved better in culture. Sprouts formed from the buds in 1 week were disinfected in ethanol 75% (v/v) (30 s) and then in a commercial bleach solution (Neoblanc, chlorine concentration 5% (v/v)) 20% (v/v) with Teepol 0.01% (v/v) (15 min). Sprouts were then rinsed in sterile water three times and sectioned in 2 - 3 cm segments that contained the shoot apex and 1 - 2 nodes. Shoot proliferation was tested in woody plant medium (Huetteman and Preece, 1993). Data were recorded 5 weeks after *in vitro* introduction. The *in vitro* rooting assay was performed using 3 - 4 cm long shoots obtained from 18 month old *L. japonica* on WPM medium. Eight variant formulations of WPM culture medium were tested (Table 2). Shoots were cultivated in WPM basal medium without growth regulators for 4 weeks (control conditions), this was followed by transference to WPM medium with different combination of auxin during 4 weeks. One shoot was placed per culture tube and cultures were grown under Osram cool white fluorescent lamps providing a light intensity of 250 µmol m^{-2} s^{-1} with a 16 h daylight period at 24°C. After five weeks, the number of shoots that regenerated roots, the number of regenerated roots per shoot and the size and morphology of root were recorded.

In order to establish a system which could be utilized for continuous microplant production and subculturing, combinations of NAA or IAA and BA were tested for their ability to multiply *in vitro* of *L. japonica*. After 90 days, the calli was transferred to regeneration media (NAA and BA combinations, IAA and BA combinations) (Table 3). During this study all media were autoclaved at 121°C and 101 kPa for 20 min after adjustment of the pH to 5.8 with 1 M NaOH. After cooling, 25 ml of the medium was poured into 50 ml conical flask. The calli were transferred to a 26 - 28°C growth room with 16 h light illumination (250 µmol $m^{-2}s^{-1}$ photosynthetic photon density) and 8 h dark. The light was provided by 'cool-white' fluorescent tubes (40W/220V×6). Observations were recorded on mean number of shoot per explant, rooting percent and survival percent after 12 weeks.

The fourth assay was completed by inducing plants direct rooting in soil with growth regulator; there 15 cm long shoots from different stems were used for direct acclimatization in soil mixture. Given their characteristics, shoots were divided in three groups: one year shoot, two years shoot and three years shoot. The shoot apex and the basal leaves were cut off and the shoots of each group had their basal end immersed in different solution during 8h (Table 3). Results were recorded after five weeks. The acclimatization assay was performed in this work. After rooting, single plant was transferred to 500 ml pots, with an autoclaved soil mixture of soil: plant ash: peat (10:2:3; wt.) which were placed in a glass chamber located in the greenhouse. Plants in pots were fogged with a 1 gl^{-1} Benlate solution. In the first week, plants were also fogged with sterile water to avoid leaf fade. In the greenhouse, incandescent light gave a light intensity of 500 µmol m^{-2} s^{-1} with a 16 h daylight period. Temperature was 26 ± 2°C and relative humidity about 85%. The results were recorded after five weeks.

Statistical analysis

All the percentage values were arcsine transformed and counts of shoots and roots were subjected to square root transformation. Results were statistically tested using a two-sided t-test after analysis of variance (ANOVA) performed by Fisher's test (Dytham, 2011). Statistical significance was assumed at $P \leq 0.05$. Results were processed using the program Microsoft Excel 2003.

Table 1. Number of axillary shoots produced from tip segments of five different species of *Lonicera.*

Species	Shoots per explant in WPM medium
Lonicera japonica Thunb.	3.4 ± 0.55[a]
Lonicera dasystyla Rehd.	3.26 ± 0.57[ab]
Lonicera confusa (Sweet) DC.	2.58 ± 0.32[b]
Lonicera hypoglauca Miq.	2.04 ± 0.21[b]
Lonicera macranthoides Hand. -Mazz.	1.65 ± 0.64[c]

Values are the average mean ± standard error of two experiments registered at 5 weeks. Values followed by a similar letter are not significantly different(P >0.05).

Table 2. Rooting of *Lonicera japonica* Thunb. from dormant buds on different WPM medium with different concentrations of IBA and IAA for five weeks.

Combination of auxin (µM)	Percentage of root production (%)	Mean number of roots per plantlet	Root length (cm)
2.0 IBA + 0.0 IAA	87	3 ± 1.58	2.22 ± 1.26[c]
4.0 IBA + 0.0 IAA	83	3 ± 1.55	2.75 ± 1.44[c]
0.0 IBA + 2.5 IAA	87	3 ± 2.68	3.01 ± 1.52[b]
0.0 IBA + 5.5 IAA	88	2 ± 1.22	2.82 ± 1.32[c]
2.0 IBA + 2.5 IAA	95	4 ± 1.38	2.55 ± 0.47[c]
3.0 IBA + 5.5 IAA	89	3 ± 2.62	3.61 ± 1.56[a]
2.0 IBA + 5.5 IAA	91	3 ± 2.02	3.42 ± 0.87[a]
3.0 IBA + 2.5 IAA	93	3 ± 2.38	2.49 ± 1.04[c]

Values are the standard error and treatments denoted by the same letter in a column were not different (P ≤ 0.05) using the LSD test. Ten replicates were used per treatment and experiments were repeated three times.

Table 3. Results of differentiation and rooting of *Lonicera japonica* Thunb. from calli using different media with the basal composition of WPM.

Combination of NAA, IAA and BA (µM)	Shoots per explant	Rooting percent	Survival percent
9.0 NAA +2.0 BA	5.50 ± 0.56[a]	84 ± 4.52[a]	75 ± 5.26[a]
6.0 NAA +4.5 BA	5.32 ± 0.66[a]	85 ± 5.45[a]	73 ± 6.53[a]
2.0 NAA +8.5 BA	5.24 ± 0.52[ab]	82 ± 7.20[a]	74 ± 6.40[a]
9.0 IAA +2.0 BA	5.00 ± 0.65[b]	82 ± 5.35[a]	71 ± 7.33[a]
6.0 IAA +4.5 BA	2.45 ± 0.36[c]	79 ± 3.71[a]	65 ± 5.35[b]
2.0 IAA +8.5 BA	3.15 ± 0.26[c]	77 ± 4.43[a]	65 ± 3.64[b]

Values are the average mean ± standard error of experiments registered at 12 weeks. Values followed by a similar letter are not significantly different (P >0.05).

RESULTS

From the assay carried out to test the genotype influence in culture, the genus *L. japonica* was the one that showed the best *in vitro* shoot production (Table 1); 3.4± 0.55 shoots were produced per explant, the difference being statistically significant (P ≤ 0.05). Therefore, *L. japonica* Thunb. was chosen for subsequent experiments of *in vitro* rooting and acclimatization. Shoots formed roots in all *in vitro* tested media. There were no significant differences between the number of roots per shoot produced in media with IBA and IAA (Table 2), however, a significantly higher number of roots per shoot (4.00 ± 1.38) were induced in WPM medium with 2.0 µM IBA and 2.5 µM IAA. WPM medium with 5.5 µM IAA promoted the lowest number among all media. Morphologically, roots were very variable, roots produced in media with 2.0 µM IBA and 2.5 µM IAA were thicker and shorter (2.0 - 3.0 cm) than roots formed in media with 3.0 µM IBA and 5.5 µM IAA (3.0 - 5.0 cm). The mean number of roots per plantlet was 2 to 4; there were no significant differences in all media. For the plants of *L. japonica* Thunb. rooted

Table 4. Percentage of *Lonicera japonica* Thunb. shoots directly acclimatized in soil mixture after immersion their basal end in different solutions.

Type of shoot	Water(control)	IBA (10 mgL^{-1})	IBA (10 mgL^{-1}) + IAA (10 mgL^{-1})	GA_3 (10 mgL^{-1}) + IBA (10 mgL^{-1})
One year shoot	58.2 ± 6.55^{bb}	64.4 ± 7.44^{bb}	66.5 ± 6.47^{bb}	72.7 ± 7.33^{ba}
Two years shoot	80.5 ± 8.42^{ab}	83.4 ± 5.63^{ab}	84.2 ± 7.55^{ab}	88.7 ± 5.84^{aa}
Three years shoot	78.5 ± 8.42^{ab}	80.4 ± 5.63^{ab}	83.5 ± 7.55^{ab}	83.7 ± 5.84^{aa}

Values are the average mean± standard error of experiments registered at 5 weeks. Values followed by a different letter, in the same column, are significantly different ($P \leq 0.05$). In the same row, the second same letters indicate values not significantly different ($P > 0.05$), the second different letters indicate values significantly different ($P \leq 0.05$).

in vitro that were transferred to soil mixture, 85.7% were totally established after 4 weeks. No morphological differences were found between these plants and the mother plant. Moreover, all media tested for microplant production and *in vitro* rooting provided obvious results. When 9.0 µM NAA was combined with BA at 2.0 µM, shoot induction differed from the other combinations tested with an average of 5.50 shoots being produced (Table 3), but their effect on rooting was statistically similar to the other IAA and BA combinations. This may imply that for shoot regeneration, the ratio of NAA or IAA to BA is important for successful plantlet regeneration. However, the rooting does not appear to be dependent on the auxin: cytokinin ratio. There were significant differences with respect to the survival percent, but the result was in accord with shoot multiplication. Furthermore, we compared the rooting percentages of *L. japonica* Thunb. shoots of different ages. There were significant differences in different types of shoot (Table 4); the two years old shoot rooted better than one year shoot, using two years old shoots the rooting percentage was above 80%, but it was statistically similar to the three years shoot. The rooting percent of shoot was only slightly increased with auxin treatments than immersion of their basal end in water, but there were significant differences when immersing them in GA_3 solution. This suggested that the addition of auxins is beneficial to rooting and the using of GA_3 is essential. This may imply that for shoot regeneration, the ratio of NAA or IAA to BA is important for successful plantlet regeneration. However, the rooting does not appear to be dependent on the auxin: cytokinin ratio.

DISCUSSION

Direct acclimatization of shoots (Table 4) resulted in a high establishment percentage particularly for shoots with two years shoot, above 80%. This was the case for three years shoot as well as two years shoot. However, taking three years shoot could damage the mother plant badly. In each kind of shoots, there were no significant differences between shoots treated with auxin and water (control), and GA_3 showed an important influence on direct rooting. Morphologically, there were no differences between the plants produced and the mother plant. After three months in the greenhouse, plants from direct rooting and *in vitro* rooting were transferred to the field and all the plants survived (Figure 1H). Morphologically, there were no differences between the plants produced and the mother plant.

Most reports on *L. japonica* micropropagation do not refer to the acclimatization process or they only mention that the acclimatization was tested with success. In our study, acclimatization was given particular attention, and two approaches were assayed: *in vitro* rooting prior to transfer to soil mixture (Figure 1B and D) and direct transfer of shoots to soil mixture (Figure 1E). Our results led us to the conclusion that formation of *in vitro* roots prior to acclimatization is not needed. This result may be due to mechanical damage of roots during transfer of plants to soil (Figure 1F). Furthermore, Bonga and Von Aderkas (1992) mention that for many hard woods, roots formed *in vitro* had enlarged cortical cells and underdeveloped vascular systems, and thus were of poorer quality than roots formed *ex vitro*. The same conclusion was drawn by Cheng and Shi (1995) and Paula et al. (2008) who reported that the *in vitro* rooting stage could waste time and cost.

In this study, we found that shoots with thin stems tolerated the stress of acclimatization better than *in vitro* rooted plants. Direct acclimatization is easy and inexpensive and allows an efficient and fast establishment of plants in soil. A group of plants was planted in the field in Hunan, Western China and they all survived (Figure 1H); they were similar to their progenitors, in relation to size and morphology of the leaves and thickness and elongation of the stem.

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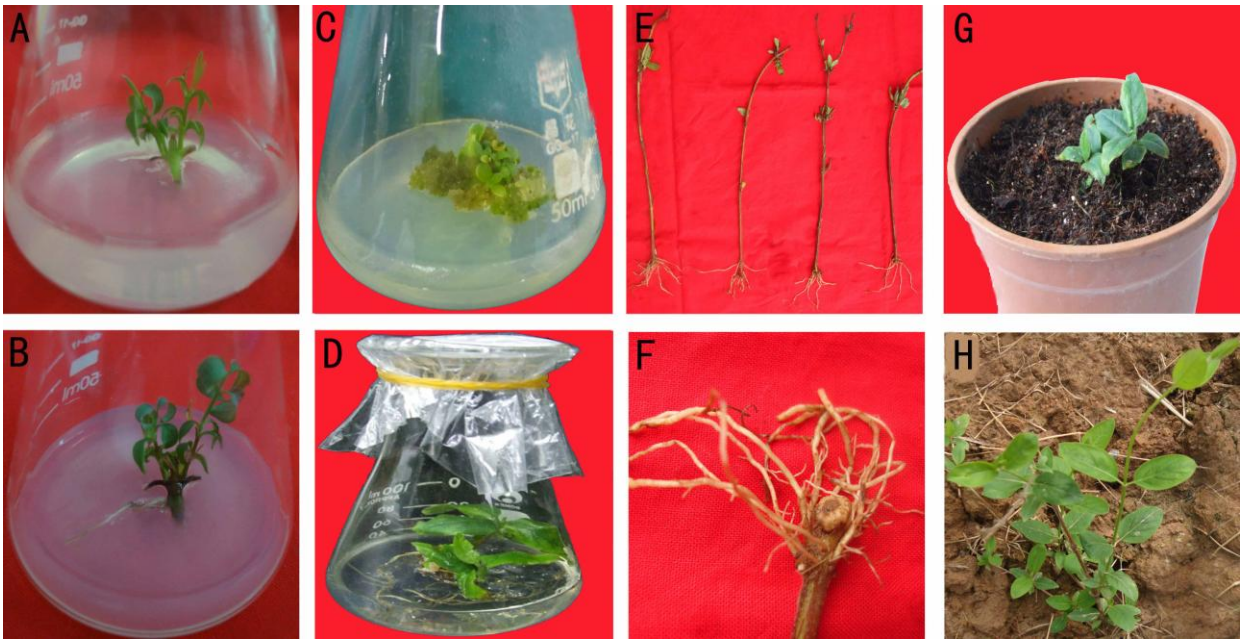

Figure 1. Comparative of different methods of micropropagation and acclimatization for *Lonicera japonica* Thunb. (A) Formation of sprouts from dormant buds collected in the field from adult plant. (B) Differentiation of callus by medium with 3.0 µM IAA and 2.0 µM BA. (C) Shoot organogenesis induced by WPM medium with 9.0 µM NAA and 2.0 µM BA. (D) Rooting situation of multiplication seedling in WPM medium with 9.0 µM NAA and 2.0 µM BA after 12 weeks. (E) Type of shoots used in direct Acclimatization in soil mixture and situation of rooting. (F) The rooting situation of shoots used in direct Acclimatization by immersed in GA_3 solution. (G) Acclimatized plants observed 6 weeks after their transfer from *in vitro* rooting medium to soil mixture. (H) *Lonicera japonica* Thunb. photographed 1 year after being planted in the field.

REFERENCES

Bonga JM, Von Aderkas P (1992). Clonal propagation. In: Bonga J M, Von Aderkas P (eds) *In vitro* culture of trees. Kluwer Academic Publishers, Boston. 72-125.

Chang WC, Hsu FL (1992). Inhibition of platelet activation and endothelial cell injury by polyphenolic compounds isolated from *Lonicera japonica* Thunb. Prostag Leukotr Ess. 45:307-312.

Cheng ZM, Shi NQ (1995). Micropropagation of mature Siberian elm in two steps. Plant Cell Tiss. Org. 41:197-199.

Georges D, Chenieux JC, Ochatt SJ (1993). Plant regeneration from aged-callus of the woody ornamental species *Lonicera japonica* cv. "Hall's Prolific". Plant Cell Rep. 13:91-94.

Dytham C (2011).Choosing and using statistics, a biologist's guide, Blackwell Publishers, UK, pp 278-320.

Houghton PJ, Boxu Z, Xi SZ (1993). A clinical evaluation of the Chinese herbal mixture "Aden-I" for treating respiratory in fections. Phytothera. Res. 7:384-386.

Huetteman CA, Preece JE (1993). Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. 33:105-109.

Cambecedes J, Duron M, Decourtye L (1991). Adventitious bud regeneration from leaf explants of the shrubby ornamental honeysuckle, *Lonicera nitida* Wils. cv. 'Maigriin': effects of thidiazuron and 2,3,5-triiodobenzoic acid. Plant Cell Rep. 10:471-474.

Kim SY, Kim JH, Kim SK, Oh MJ, Jung MY (1994). Antioxidant activities of selected oriental herb extracts. Jam Oil Chem. Soc. 71:633-640.

Lanteri R, Acquaviva R, DiGiacomo C, Sorrenti VL, Destri G., Santangelo M, Vanella L, DiCataldo A (2007). Rutin in rat liver ischemia/reperfusion injury: effect on DDAH/NOS pathway. Microsurgery 27:245-251.

Lao CJ, Lin JG, Kuo JS, Chao PD, Cheng CY, Tang NY, Hsieh CL (2005). Microglia, apoptosis and interleukin-1beta expression in the effect of *Sophora japonica* on cerebral infarct induced by ischemia-reperfusion in rats. Am. J. Chinese Med. 33:425-438.

Lee JH, Ko WS, Kim YH, Kang HS, Kim HD, Choi BT (2001). Anti-inflammatory effect of the aqueous extract from *Lonicera japonica* flower is related to inhibition of NF-kappaB activation through reducing I-kappaBalpha degradation in rat liver. Int. J. Mol. Med. 7:79-83.

Palacios N, Christou P, Lee MJ (2002). Regeneration of *Lonicera tatarica* plants via adventitious organogenesis from cultured stem explants. Plant Cell Rep. 20: 808-813.

Paula C, Alexandra S, Armando C, Conceicao S (2008). A protocol for *Ulmus minor Mill*. micropropagation and acclimatization. Plant Cell Tiss. Org. 92:113-119.

Pharmacopoeia of the People`s Republic of China (2005). Chemical Industry Press, Peking, p. 152.

Son KH, Jung KY, Chang HW, Kim HP, Kang SS (1994). Triterpenoid saponins from the aerial parts of *Lonicera japonica*. Phytochemistry. 35:1005-1008.

Zenk MH (1980). Enzymatic synthesis of ajmalicine and related indole alkaloids. J. Nat. Prod. 43:438-451.