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In vitro Plantlet Regeneration of *Abies firma* from Germinated Seedlings

L-M. VAARIO*, Megumi TANAKA* and Yuji IDE**

Introduction

The genus *Abies* (Pinaceae) is widely distributed throughout Europe, North Africa, northern and central Asia, and North America. It includes over 40 species (Record and Hess, 1943) all of which form mycorrhizal associations (Meyer, 1973).

Abies species are sensitive to environmental change such as drought, low potassium levels and air pollution (Požgaj *et al.*, 1996; Freer-Smith, 1996). *Abies* forest decline occurs in both Asia (Donaubauer, 1993) and central Europe (Freer-Smith, 1996), and is intensifying (Kandler, 1993). Mycorrhiza are considered to be an important factor associated with forest decline (Metzler and Metzler, 1992). However, there are fewer physiological studies from the viewpoint of mycorrhizal association on this genus than on other genera belonging to Pinaceae, such as *Pinus* and *Picea*.

Seedlings of *Abies* species grow slower than other Pinaceae species, thus limiting mycorrhizal research on the genus. *In vitro* culture techniques are capable of promoting root development and can be used to study the interaction between mycorrhizal fungi and hosts (Bonga and von Aderkas, 1992). The establishment of a tissue culture system will allow physiological research on mycorrhizal associations.

While there are numerous reports on *in vitro* culture of coniferous species (Park and Bonga, 1993), studies on *Abies* species are limited (Saravitz and Blazich, 1996). Early experiments on organogenesis are reported on *Abies balsamea* (Bonga, 1977) and *Abies fraseri* (Saravitz *et al.*, 1991) with limited success. Recently, somatic embryo formation was reported on hybrid firs (*Abies alba*×*Abies cephalonica* and *A. alba*×*Abies numidica*; Salajova *et al.*, 1996) and *A. fraseri* (Guevin and Kirby, 1997).

Abies firma Sieb. et Zucc. (Japanese fir, momi fir) is a species endemic to warm temperate forests of Japan, forming natural coniferous forests with *Tsuga sieboldii* Carr. (Ishizuka, 1974). While *A. firma* is not so commercially important today, it is one of the indicators of sound forest vegetation in the warm Temperate Zone of Japan. However, there is little research on mycorrhiza physiology and no reports of *in vitro* culture of this species.

The aim of this research is to establish *in vitro* culture systems of *A. firma* feasible for physiological research on mycorrhizal associations. Here, we report on root regeneration from seedlings from which radicles were removed to form highly developed root systems available for mycorrhiza formation and on adventitious bud formation from an excised hypocotyl for providing cloned material for physiological research.

Material and Methods

Plant material and surface sterilization

Seeds of *A. firma* were collected in a natural stand in The University Forest in Chiba, The University of Tokyo, at the end of July 1997. They were air-dried and stored in a

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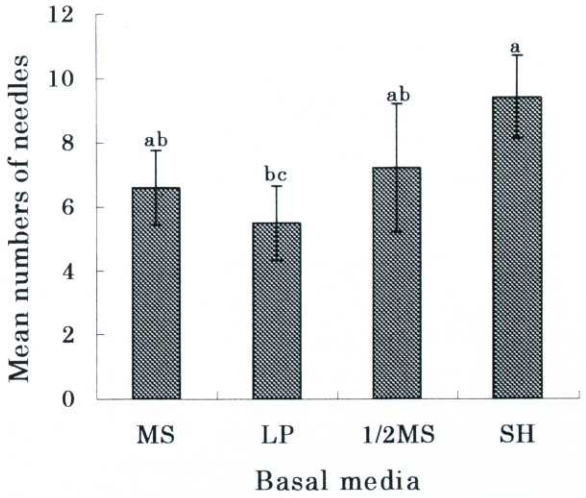

Fig. 1. Effect of different basal media on needle proliferation of de-rooted seedlings of *Abies firma* after 5 weeks of culture. The basal medium contained 1.0 mg/*l* BAP and 0.1 mg/*l* NAA. Different letters over bars represent significant differences according to Tukey test at the 0.05 level of probability. Each column indicates standard deviation of means (n=20).

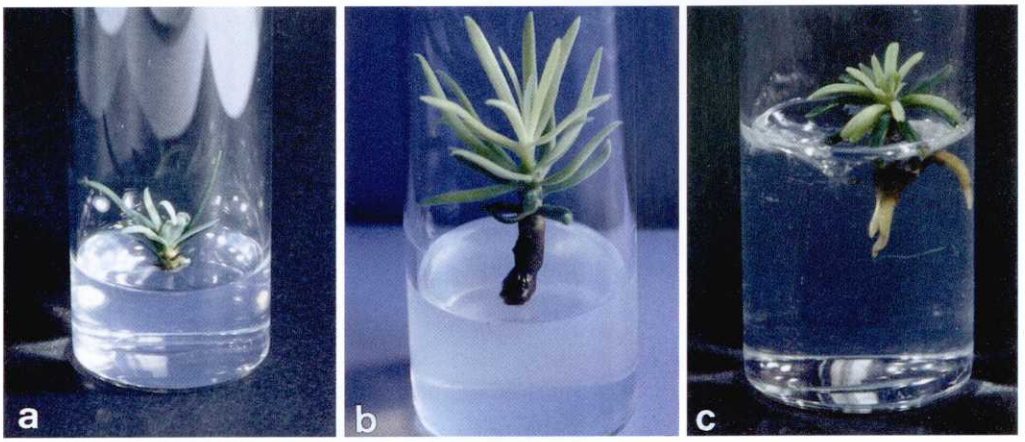

Fig. 2. Plantlet regeneration from de-rooted seedlings in *Abies firma*.
a. Growth of de-rooted seedlings on SH medium containing 1.0 mg/*l* BAP and 0.1 mg/*l* NAA after 4 weeks of culture. b. Elongation of de-rooted seedlings on hormone-free SH medium after 9 weeks of culture. c. Root formation of de-rooted seedlings after 6 weeks of culture on 1/2SH containing 0.1 mg/*l* BAP, 0.1 mg/*l* 2ip, 0.3 mg/*l* NAA and 0.3 mg/*l* IAA with immersion in 1000 ppm NAA.

polyethylene bag at 4℃ until sowing. Seeds were sown in vermiculite after immersion in 1/2000 benomyl (Benlate®; Dupon, U.S.A.) for 1 day. They were germinated at room temperature under diffuse fluorescent illumination.

Three-week-old and 10-day-old seedlings with cotyledons, 4–5 cm hypocotyls and radicles, were used for *in vitro* cutting and adventitious bud formation respectively. They

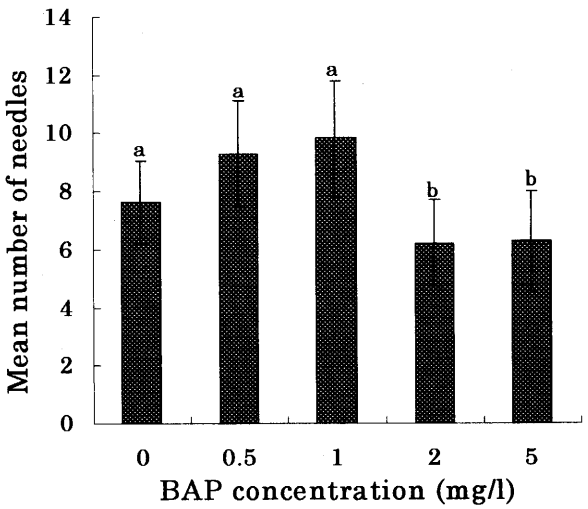

Fig. 3. Effect of different BAP concentrations on needle proliferation of excised hypocotyls of *Abies firma* after 5 weeks of culture (the basal medium was SH medium containing 0.1 mg/*l* NAA and BAP). Different letters over bars represent significant differences according to Tukey test at the 0.05 level of probability. Each column indicates standard deviation of means (n=20).

Table 1. Effect of growth regulators on callus differentiation from hypocotyl segments of *Abies firma* after 5 weeks of culture

NAA (mg/*l*)	BAP (mg/*l*)			
	0.1	0.3	1.0	3.0
0.01	32*	25	58	67
0.03	72	67	88	33
0.1	61	75	75	50
0.3	58	50	83	33

* Frequency of callus formation (%)

were soaked in 70% (v/v) ethanol for 1 min and in sodium hypochlorite solution containing 1% (v/v) active chlorine for 10 min successively. Following three rinses in sterile deionized water, they were soaked in 0.05% (w/v) mercuric chloride solution for 6 min and rinsed four times in sterile deionized water.

In vitro rooting of de-rooted seedlings

Radicles were removed from surface sterilized seedlings. These de-rooted seedlings were transferred to solid agar media.

For the screening of an appropriate basal medium, MS medium (Murashige and Skoog, 1962), 1/2MS medium (concentrations of macro elements of MS medium were halved), SH medium (Schenk and Hildebrandt, 1972) and LP medium (von Arnold and Eriksson, 1977) were used. They were supplemented with 1.0 mg/*l* BAP (6-benzylaminopurine) and 0.1 mg/*l* NAA (α-naphthylacetic acid). Explants were also cultured on SH medium supplemented with 0, 0.5, 1.0, 2.0 and 5.0 mg/*l* BAP, and 0.1 mg/*l* NAA to determine the optimum BAP

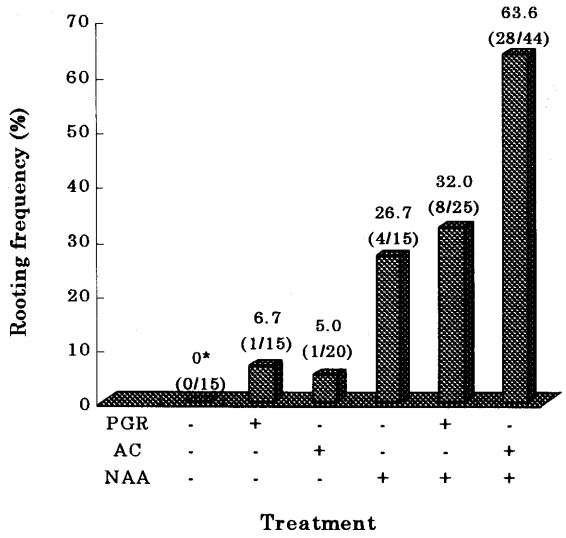

Fig. 4. Effect of different treatments on rooting of de-rooted seedlings of *Abies firma*. (NAA indicates 1,000 ppm NAA solution pretreatment for 1 min; AC indicates the basal medium containing 0.3% activated charcoal; PGR indicates the basal medium containing plant growth regulators: 0.1 mg/*l* BAP, 0.1 mg/*l* 2ip, 0.3 mg/*l* NAA and 0.3 mg/*l* IAA; *figures indicates the number of rooted explants/tested explants).

concentration. Twenty explants were used for each treatment, respectively.

Explants which developed more than four needles were again cut at their hypocotyl ends and placed on rooting medium. The cut ends were immersed in 1,000 ppm NAA solution for 1 min before incubation. 1/2SH medium, in which all components of SH medium were reduced to 50% concentration, was used as the rooting medium. It was supplemented with 0.3 mg/*l* IAA (β-indoleacetic acid), 0.3 mg/*l* NAA, 0.1 mg/*l* BAP, 0.1 mg/*l* 2-ip (2-isopentenyladenine) and 0.3% activated charcoal (Wako Pure Chemical Industries, Japan) in various combinations (Fig. 4).

Plant regeneration by adventitious bud formation

Hypocotyl segments about 1 cm long were excised from the surface sterilized seedlings and placed on SH medium containing 0.1, 0.3, 1.0 and 3.0 mg/*l* BAP, and 0.01, 0.03, 0.1 and 0.3 mg/*l* NAA in all possible combinations. Eighteen explants were used for each treatment.

To promote shoot elongation, adventitious buds formed on SH medium were transferred to hormone-free 1/2SH medium. The elongated shoots were removed from the explants and their cut ends were immersed in 1,000 ppm NAA solution for 1 min. Then they were transplanted to 1/2SH medium containing 0.3% activated charcoal.

All media used in this experiment contained 20 g/*l* sucrose and were solidified with 0.3% (w/v) Gelrite (Wako Pure Chemical Industries, Japan). The pH of each medium was adjusted to 5.6 prior to autoclaving for 20 min at 121℃. Cultures were incubated under 3,000 lux fluorescent light with a 16 h photo period at 24±2℃.

Results

In vitro rooting of de-rooted seedlings

As shoot elongation is very slow in *A. firma in vitro* culture, the number of newly developed needles (NDN) was used as an indicator of growth.

The mean number of NDN on different basal media after 5 weeks of culture is shown in Fig. 1. More than nine needles on average formed on SH medium, which was the best result for needle development among tested basal media. Therefore, average NDN on SH medium was significantly higher than that on LP medium according to t-test ($p < 0.005$). There was no significant difference between SH medium and MS or 1/2MS medium (Fig. 1).

Explants cultured on LP and MS medium gradually turned brown within 3 weeks, however explants cultured on SH medium remained green throughout the culture period (Fig. 2a, b). Accordingly, SH medium was selected for further studies.

The addition of BAP at concentrations of 0.5 mg/l and 1.0 mg/l stimulated the development of new needles and the number of NDN was maximized at a concentration of 1.0 mg/l (Fig. 3). However, there was no significant difference among average number of NDN at concentrations from 0 to 1.0 mg/l by t-test. Concentrations of 2.0 and 5.0 mg/l BAP suppressed needle development. The mean number of NDN on these two media was less than the medium without BAP, and the explants cultured on these media appeared yellow in color. On the other hand, callus formation occurred at the bases of more than 85% of the de-rooted seedlings cultured on BAP-containing media, compared with only 10% of explants forming callus on the media without BAP.

After 5 weeks of culture explants which developed more than four needles were cut at their hypocotyl end again and placed on rooting medium following immersion in 1,000 ppm NAA solution for 1 min. The first rooted explant was observed on 1/2SH rooting medium containing 0.3 mg/l IAA, 0.3 mg/l NAA, 0.1 mg/l BAP and 0.1 mg/l 2ip 4 weeks after transplantation. The roots were induced directly from the cut ends of de-rooted seedlings (Fig 2c). Explants of which the cut ends were immersed in 1,000 ppm NAA solution for 1 min prior to inoculation showed remarkably higher frequencies of rooting than the explants without NAA treatment. After 6 weeks of culture, the rooting frequency of NAA-treated explants reached 63.6% in the media containing activated charcoal (Fig. 4) and they averaged 4 regenerated roots. Addition of BAP, 2ip, NAA and IAA in combination, or activated charcoal only, had a small effect on rooting. In the media containing plant growth regulators, the frequency of rooted explants was 32%. However most roots were formed on the swollen bases of the explants, and they readily turned brown.

Plant regeneration by adventitious bud formation

Hypocotyl explants turned light green during 2 weeks of culture. A slight swelling of the explants occurred after 3 weeks on every combination of BAP and NAA (Fig. 5a). These swellings developed into callus after 5 weeks of culture. The greatest rate of callus formation was recorded on the medium containing 1.0 mg/l BAP and 0.03 mg/l NAA (Table 1). Only on the medium containing 1.0 mg/l BAP and 0.3 mg/l NAA, the swellings developed into a mass of meristematic tissue after 5 weeks of culture (Fig. 5b).

The explants forming meristematic tissues were transplanted to hormone-free 1/2SH medium to promote adventitious shoot elongation. After 3 weeks culture on hormone free medium, meristematic tissue developed into shoots with about 8 needles (Fig. 5c). Then shoots were cut from the explants and transferred to 1/2SH medium containing 0.3% activated charcoal following pretreatment with 1,000 ppm NAA solution for 1 min. The adventitious shoots rooted within 6 weeks of culture on rooting medium and regenerated

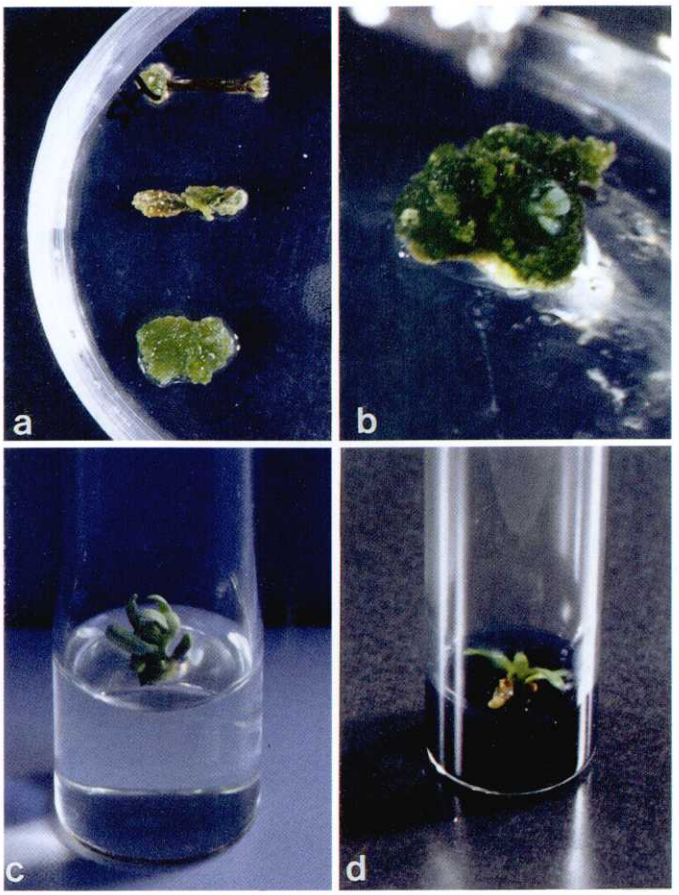

Fig. 5. Plantlet regeneration from hypocotyl segments of *Abies firma*.
a. Callus formation from hypocotyl segments incubated on SH medium containing 1.0 mg/*l* of BAP and 0.3 mg/*l* of NAA after 3 weeks of incubation. b. Meristematic tissue appeared on the surface of callus after 6 weeks of incubation. c. Development and elongation of adventitious shoot on 1/2SH medium after 7 weeks of incubation. d. Root formation of adventitious shoot on 1/2SH medium containing 0.3% activated charcoal after 8 weeks of incubation.

complete plantlets (Fig. 5d).

Discussion

The four basal media tested showed different effects on new needle development of de-rooted seedlings of *A. firma*. SH medium was the most effective among tested media. Salajova et al. (1996) showed SH medium supplemented with 1.0 mg/*l* BAP to be the most effective for callus induction of hybrid firs (*A. alba*×*A. cephalonica* and *A. alba*×*A. numidica*) and its maintenance. SH medium was also selected as the most appropriate medium for embryogenic suspension cultures of *A. alba* by Hartmann *et al.* (1992). Therefore, we can conclude SH medium is an appropriate medium for *in vitro* culture of *Abies* species.

The greatest number of NDN was achieved on the medium containing 1.0 mg/*l* BAP

and was markedly suppressed on media containing more than 2.0 mg/*l* BAP. However, there were no significant differences among the media containing 0, 0.5 and 1.0 mg/*l* BAP. Over 85% of explants formed callus at the cut bases of hypocotyls on BAP-containing media. Elongation of the hypocotyls seemed to be inhibited by callus formation (data not shown). These results suggest that BAP is not important in promoting the elongation of hypocotyls of de-rooted seedlings. Schuller *et al.* (1989) found that embryos of *A. alba* only developed and formed cotyledons on SH medium without BAP. Therefore, we considered that BAP is not effective for organ development in *Abies* species.

For successful *in vitro* rooting of de-rooted seedlings of *A. firma*, pretreatment of the cut ends of the explants with 1,000 ppm NAA solution for a short period (1 min) was essential. Saravitz *et al.* (1990) also reported the necessity of auxin pretreatment for *in vitro* hypocotyl cuttings of *A. fraseri*. Addition of activated charcoal to the medium was also effective in preventing browning of roots probably caused by phenolic oxidation of the tissue (Preece and Compton, 1991). Browning of tissue affects plant metabolism in various ways. Rumary and Thorpe (1984) also reported the positive effect of activated charcoal on the rooting of both black and white spruce.

In this experiment, well developed multiple roots were formed on the medium containing activated charcoal following NAA pretreatment. By this procedure, aseptic plantlets having well-developed root systems will be provided for experimentation on *in vitro* establishment of mycorrhizas.

Adventitious bud formation occurred successfully from hypocotyl segments. However, it only occurred on the medium containing a combination of 1.0 mg/*l* BAP and 0.3 mg/*l* NAA. Saravitz *et al.* (1991) found those 12-day-old hypocotyls of *A. fraseri* had begun to loose regenerative capacity. Hypocotyls excised from seedlings older than 10 days could not regenerate any adventitious buds under the same condition described above in *A. firma* (data not presented). The effect of age on explant adventitious bud differentiation should be examined.

The adventitious buds developed into shoots on hormone-free 1/2SH medium. The shoots rooted on hormone-free 1/2SH medium containing activated charcoal after immersion in 1,000 ppm NAA solution indicating that the rooting method established in this experiment on the *in vitro* rooting of de-rooted seedlings is effective for the rooting of *A. firma* in general.

Plant regeneration through adventitious bud formation will provide numerous cloned plants. They will then be useful for the *in vitro* evaluation of the mycorrhizal effects on plant physiology.

Acknowledgment

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Summary

Two successful plant regeneration procedures for *Abies firma* from seedlings from which radicles were removed and from excised hypocotyl segments through adventitious bud formation were established. SH medium was found to be adaptable to needle development of de-rooted seedlings. They developed the maximum number of new needles on SH medium containing 1.0 mg/*l* BAP and rooted on hormone-free 1/2SH medium containing 0.3% activated charcoal following immersion in 1,000 ppm NAA solution for 1 min. The

frequency of rooted explants was 63.6%. Adventitious buds were induced on excised hypocotyls on SH medium supplemented with 1.0 mg/*l* BAP and 0.3 mg/*l* NAA after 5 weeks culture. Elongation of adventitious buds was promoted on hormone-free 1/2SH medium. Regenerated plantlets were successfully obtained by the procedure used in the rooting of de-rooted seedlings as described above.

Key words: *Abies firma*, plant regeneration, *in vitro* culture, hypocotyl, adventitious bud

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In vitro におけるモミ実生からの植物体再生

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要 旨

モミ (*Abies firma*) について，幼根を除去した芽生えの発根および下胚軸片からの不定芽形成による植物体再生法を確立した。幼根を除いた芽生えの針葉の形成には SH 培地が適していた。特に，1.0 mg/*l* の BAP を添加した SH 培地で新たな針葉形成が最大となった。新たな針葉の展開した芽生えは 1,000 ppm の NAA 水溶液に基部を 1 分間浸漬した後，0.3% の活性炭を添加したホルモンを含まない 1/2SH 培地に移植したところ，発根して植物体が再生した。発根率は 63.6% であった。培養 5 週間後に，BAP 1.0 mg/*l* と NAA 0.3 mg/*l* を添加した SH 培地上で培養した下胚軸片から，不定芽が形成された。これらをホルモンを含まない 1/2SH 培地に移すシュートの形成が見られ，伸長したシュートは幼根を幼根を除いた芽生えの場合と同じ方法で発根させ植物体を再生させることが出来た。

キーワード: モミ (*Abies firma*)，植物体再生，組織培養，下胚軸，不定芽

In vitro Plantlet Regeneration of *Abies firma* from Germinated Seedlings

L-M. VAARIO, Megumi TANAKA and Yuji IDE

Plant regeneration procedures from seedlings without radicles and from hypocotyl segments through adventitious bud were established. De-rooted seedlings developed the maximum number of new needles on SH medium containing 1.0 mg/*l* BAP and rooted on hormone-free 1/2SH medium containing 0.3% activated charcoal after immersion in 1,000 ppm NAA solution. The frequency of rooting was 63.6%. Adventitious buds were induced on hypocotyl segments on SH medium with 1.0 mg/*l* BAP and 0.3 mg/*l* NAA. They grew into shoots on hormone-free 1/2SH medium. Plantlets were successfully obtained by the procedure used in the rooting of de-rooted seedlings.

Studies on Yarding and Hauling System of Mobile-yarder, Processor, and Forwarder with Simulation Methods

Rin SAKURAI, Masahiro IWAOKA, Hideo SAKAI and Hiroshi KOBAYASHI

The length of the skidding road to minimize the skidding cost, for mobile-yarder, processor and forwarder systems were studied by simulation models involving the number of operators and other factors. Simulation is based on a model skidding area of 100 m×60 m. If the single-grip type processor is used, distance from site to mobile-yarder should be 0 m–40 m with three operators. For a simple type processor, if the site is close to the forest road, distance from site to mobile-yarder should be 0 m–40 m with three operators. If distance are greater, the profitable length of skidding road should be short as possible with four operators.

Anti-Bioluminescent Activity of Coniferous Bark Extracts on MICROTOX™ Test

Sakae SHIBUTANI, Masahiro SAMEJIMA, Yoshimasa SABURI and Norihisa TATARAZOKO

Anti-bioluminescent activity of bark extractives from six coniferous species was examined by MICROTOX™ test. The most active compound was purified from the *n*-hexane extracts of *A. sachalinensis* and identified as oleic acid.