Gartenbauwissenschaft, 66 (2). S. 68–71, 2001, ISSN 0016–478X. © Verlag Eugen Ulmer GmbH & Co., Stuttgart

Micropropagation of *Pinus kesiya* Royle ex Gord (Khasi pine)

Mikrovermehrung von *Pinus kesiya* Royle ex Gord (Khasi pine)

Dilip Nandwani, Suman Kumaria, and P. Tandon
(Plant Biotechnology Laboratory, Department of Botany, NE Hill University, Shillong-793014, India)

Summary

Plantlets of *Pinus kesiya* Royle ex Gord (Khasi pine) were regenerated from shoot tip explants excised from 14–20 day old seedlings. Maximum shoot y formation and growth were obtained on Murashige and Skoog (MS) medium containing 23 uM kinetin. Frequency of multiple shoot y formation was highest in MS medium followed by Schenk and Hildebrandt and Lepoivre media. Kinetin and benzyl adenine were almost equally effective. Four weeks on cytokinincontaining medium was optimal for multiple shoot y induction. Shoot elongation and multiplication was achieved on cytokininfree medium. Welldeveloped shoots were rooted on Gresshoff and Doy (GD) medium devoid of growth regulators following a 5-day exposure to 1-naphthalene acetic acid or indolebutyric acid.

Zusammenfassung

Aus Sproßspitzen von 14 bis 20 Tage altenSämlingen von Pinus kesiya werden in vitro Pflanzen regeneriert. Eine maximale Sproßbildung erfolgte auf Murashige und Skoog-Medium mit 23 µM Kinetin. Die Sproßentwicklung wurde auf cytokininfreiem Medium erzielt. Gut entwickelte Sprosse konnten nach einer fünf Tage dauernden Kultur auf Medium mit 1 NAA oder IBA und anschließender Überführung auf hormonfreies Medium erzielt werden.

Introduction

Micropropagation systems have the potential for rapidly multiplying high value genotypes for reforestation. Recent successes of plantlet regeneration in conifers through adventitious bud formation on seedling explants have progressed enormously (AHUJA, 1993; GUPTA et al, 1993, DUMAS and MONTEUUIS, 1995, FRANCLET et al., 1980, FRANCO and SCHWARZ, 1985, GOLDFARB et al., 1996, GUEVIN and KIRBY, 1997, HALOS and GO, 1993, HORGAN and AITKEN, 1981, PULIDO et al., 1992, STOJICIC et al., 1999, TUSKAN et al., 1990). *Pinus kesiya* Royle ex Gord (Khasi pine) is a commercially important conifer of Eastern India providing pulp, lumber and oleoresins. It is distributed in the Himalayan region of the Indian subcontinent. Presently, Khasi pine is used in tree improvement research and reforestation programs in North-Eastern India. The aim of the present work is to develop a technique for in-vitro shoot multiplication and plantlet regeneration from shoot tip explants.

Materials and Methods

Open-pollinated seeds from superior genotypes of Khasi pine (State Forest Department, Meghalaya) were soaked in water and placed in a refrigerator for 2 days at 4 °C for stratification. Seeds were surface disinfected with 6 % H_2O_2 (v/v) for 15 min. , rinsed in sterile water 3 or 4 times, dipped in 0.5 % aqueous HgCl2 solution (w/v) for 3 min and again washed with sterile water. Finally, seeds were transferred to growth-regulator-free MS medium (MURASHIGE and SKOOG, 1962) ingroups of 100 ml Erlenmeyer flask. Explants (cotyledons + stem apex + part of the hypocotyl) were excised from 2-to 3-week old seedlings. Basal media tested for shoot bud induction and multiplication were MS, Lepoivre (LP) (QUOIRIN and LEPOIVRE, 1977) and Schenk and Hildebrandt (SH) (SCHENK and HILDEBRANDT, 1972). Kinetin and BA were incorporated in these media at a range of concentrations for shoot regeneration. Explants were cultured for 4 weeks on cytokinin-containing MS, LP or SH media for the development of shoot buds and then transferred to the same media (i.e. MS, SH and LP) without cytokinin. Explants with multiple shoot buds were cut into 2 or 3 segments and transferred to fresh medium in 100 ml Erlenmeyer flasks every 4 weeks for elongation and multiplication of shoots. In each treatment, 24 explants were used and experiments were repeated at least twice. Data were recorded after 4 weeks. Gresshoff and Doy (GD) medium (GRESSHOFF and DOY, 1972) was used for multiplication of shoot buds and rooting experiments. Well-developed shoots (3.0–3.5 cm) were harvested from multiplication medium and placed upright in GD medium containing different concentrations (14.8–53.7 um) of NAA or IBA for root induction. Shoots were transferred to auxin-free GD medium after 24–120 h. The pH of LP and GD media was adjusted to 5.5 and of SH and MS to 5.8 with 0.1 N KOH or 0.01 N HCl before autoclaving the medium at 125 kPa (121 °C) for 20 min. Explants were cultured in 125 mm x 25 mm culture tubes or 100 ml Erlenmeyer flasks containing 20 ml and 40 ml medium, respectively, and the vessels were closed with non-absorbent cotton plugs. One or two explants were transferred in tubes and flasks, respectively. Culture vessels were incubated at 25 ±1 °C with a 16-h photoperiod (50–70 umol m^2s^2 cool white fluorescent and incandescent lamps) and 60 % relative humidity. Rooted plantlets were removed from the culture vessels and washed thoroughly in distilled water to remove any adhering agar and transferred to pots containing non-sterile soil and vermiculite (1:1 v/v) for further development.

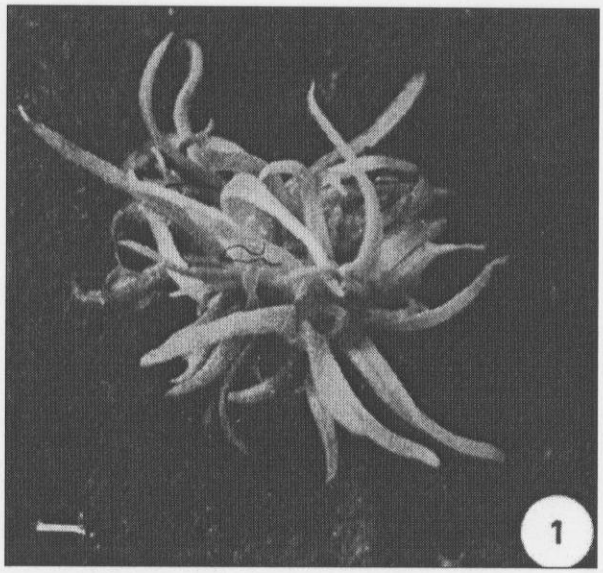

Fig. 1. Plantlet regeneration in *Pinus kesiya*
Formation of adventitious buds and needle primordia in shoot explants on MS medium containing 23 uM kinetin
Pflanzenregeneration bei P. kesiya
Bildung von Adventivsprossen und Nadelprimoordien an Sproßspitzenexplantaten

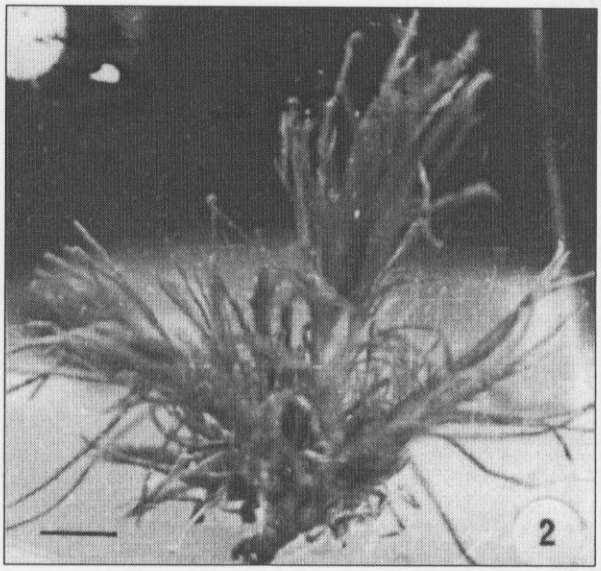

Fig. 2. Multiplication and elongation of shoots on cytokinin free MS medium after 8 weeks
Vermehrung und Sproßstreckung auf cytokininfreiem MS-Medium nach acht Wochen

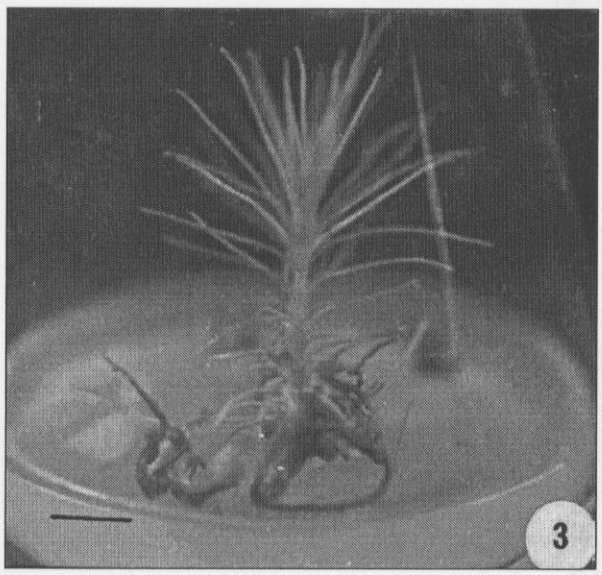

Fig. 3. Rooting in regenerated shoots on GD medium devoid of growth regulators after 3 weeks of auxin pretreatment
Bewurzelung von Sprossen auf Medium ohne Wachstumsregulatoren nach drei Wochen Vorbehandlung auf auxinhaltigem Medium

Results and Discussion

Over 80 % of the germination in seeds were observed after 8–10 days. No serious problem of contamination was encountered and microbe-free cultures were obtained. Several small and swollen bud and needle primordia appeared on the explants 4–5 day after transfer to cytokinin-containing media. Axillary buds developed more rapidly on cotyledonary nodes of the explants than adventitious buds and a proliferated leafy structure was formed in 4 weeks. After 4 weeks, cotyledonary explants formed numerous shoot buds on various media in response to different cytokinin (Table 1). Low number of shoot buds was produced in control treatments. Kinetin and BA were almost equally suitable in regeneration of shoot buds. The highest percent of shoot-forming explants was obtained on MS medium containing 23 um kinetin. Incorporation of higher amounts of cytokinin (22.2 uM BA or 23 um Kinetin) in the medium favored an increase in the number of shoot buds per explant. Shoot bud formation was obtained on LP, MS and SH media. However, MS medium was more suitable for multiple shoot bud formation than SH or LP. Shoots grown on different media did not show any morphological variation. Reasons for better growth and development of buds on MS medium might include its high level of reduced nitrogen, calcium and other salts. Cytokinin and media effects similar to the ones observed here have been reported in *Pinus elliotti* (BURNS et al 1991), *Pinus strobus* (CHESICK et al, 1991), *Pinus rigida* (PATEL et al, 1986), *Pinus heldreichii* (STOJICIC et al., 1999).

Transfer of explants grown on cytokinin-containing LP, SH or MS medium to GD medium without cytokinin resulted in multiplication and elongation of shoots. A minimum of 44 explants from each medium was transferred to GD medium. The shoot buds grew rapidly after subculture to fresh medium and highly regenerative tissues were produced in passages. Multiple shoot formation could be maintained up to eight passages on GD medium without decline in growth and deterioration in shoots. Maximum 8 well-developed leafy shoots (3.0–4.0 cm) were harvested from each explant of 60 % in total.

Shoots required exposure to NAA or IBA incorporated in GD medium for root induction and spontaneous rooting of regenerated shoots was not observed. After

exposure to 16.1 um NAA for 5 days, shoots that were at least 3.0–4.0 cm long rooted on auxin-free GD medium. Healthy roots with 5–7 laterals were produced 3 weeks after auxin pretreatment. A maximum of 67 % rooting was achieved in regenerated shoots (Table 2). IBA was less effective than NAA in root formation.

The growth of axillary buds and bud induction and development of adventitious buds in shoot tip explants of Khasi pine were similar to that observed in other pine species (BURNS et al, 1991; CHESICK et al., 1991; HALOS and GO, 1993, SOMMER et al., 1975). However, vegetative propagation of Khasi pine through cotyledonary explants has not been reported previously. The technique described here could be exploited for large- scale production of Khasi pine after further refinement of protocol.

Table 1. Influence of cytokinin and culture media on multiple shoot regeneration from shoot tip explants of Khasi pine (*Pinus kesiya*) after the first subculture of 4 weeks.
Einfluß von Cytokinin und Medium auf die multiple Sproßbildung an Sproßspitzenexplantaten von P. kesiya nach vier Wochen Kulturdauer.

Cytokinin (µM)	Media	Mean number of shoot buds/explants (±S.D)	No. of explants regenerated (%)
Control			
	LP	1.25 ± 0.24	11.1
	SH	1.16 ± 0.1	16.7
	MS	1.11 ± 0.1	12.5
BA			
4.4	LP	4.2 ± 0.3a	23.6
	SH	7.1 ± 0.54a	47.2
	MS	8.5 ± 0.44a	34.7
22.2	LP	13.6 ± 0.51a	29.1
	SH	14.8 ± 0.75a	38.8
	MS	20.6 ± 0.64a	48.6
Kinetin			
4.6	LP	20.6 ± 0.64a	43.05
	SH	5.3 ± 0.8a	37.5
	MS	9.7 ± 0.81a	47.2
23	LP	10.6 ± 0.93a	27.7
	SH	15.6 ± 0.82a	45.8
	MS	22.6 ± 0.47a	54.1

a. Data are significantly different from the control as indicated by student's t-test (p = 0.5)

Table 2. Effect of auxin on root induction in regenerated shoots of Khasi pine (*Pinus kesiya*) on GD medium. Twelve shoots were used in each treatment and experiments were repeated twice. Data were recorded 3 weeks after auxin treatment.
Einfluß von Auxin auf die Wurzelinduktion drei Wochen nach Auxinbehandlung.

Auxin	Concentration (µM)	Treatment period (h)	Shoots rooted (%)
IBA			
	14.8	24	0
	14.8	120	0
	49.2	24	0
	49.2	120	42
NAA			
	16.1	24	33
	16.1	120	67
	53.7	24	58
	53.7	120	50

ANOVA test, F value is 2.77 for the data at 5 % significance.

Financial support received from the Department of Biotechnology (DBT), Govt. of India grant no. BT/8/1/90 is gratefully acknowledged. The authors acknowledge with gratitude the assistance of Dr. V.L. Mandowara, Department of Statistics, ML Sukhadia University, Udaipur for statistical analysis. The senior author wishes to thank Dr. A.Franclet, AFOCEL, Nangis for valuable suggestions and Mrs. S. Purkayastha for technical assistance.

Literature

AHUJA, M.R. 1993: Micropropagation à la carte. In: Micropropagation of woody plants. Ahuja, M.R (ed.). Kluwer Academic Pub. Dordrecht, pp. 3–9

BURNS J.A., D.J. SCHWARZ and S.E. SCHLARBAUM. 1991: Multiple shoot production from seedling explants of slash pine (*Pinus elliotti* Engelm). Plant Cell Rep. **10**, 439–443.

CHESICK, E.E., C.A. MOHN and W.P. HACKETT 1991: Plantlet multiplication from white pine (*Pinus strobus* L.) embryos in vitro: bud induction and rooting. Plant Cell Tissue Organ Cult. **26**,107–114.

DUMAS, E. and O. MONTEUUIS 1995: In-vitro rooting of micropropagated shoots from juvenile and mature *Pinus pinaster* explants: influence of activated charcoal. Plant Cell Tissue Organ Cult. **40**, (3), 231–235.

FRANCLET A., A. DAVID, H. DAVID, and M. BOULAY 1980: Premiere mise en evidence morphologique d'un rejeunissement de meristemes primaires caulinaires de pin maritime age (*Pinus pinaster* Sol.) C.R. Acad. Sci. Paris 290: 927–930.

FRANCO E.O and D.J. SCHWARZ 1985: Micropropagation of two conifers. *Pinus oocarpa* Schiede and *Eupressus lusitanica* Miller. In: Henke R.J, Hughes K.W and A Hollander (Eds.) Tissue Culture in Forestry and Agriculture pp. 195–213. Plenum press, New York.

GOLDFARB B., G.M. HOWE, W.P.HACKETT and O. MONTEUUIS 1996: Survival and growth of eastern white pine shoot apical meristems in vitro. Plant Cell Tissue Organ Cult. **46**, (3), 171–178.

GRESSHOFF P.M. and C.H. DOY 1972: Development and differentiation of haploid *Lycopersicon esculentum* (tomato). Planta **107**, 161–170.

GUEVIN, T.G and KIRBY, E.G. 1997: Induction of embryogenesis in cultured mature zygotic embryos of *Abies fraseri* (Pursh) Poir. Plant Cell Tissue Organ Cult. **49**, (3), 219–222

GUPTA P.K., G. PULLMAN, R.TIMMIS, M. KREITINGER, W.C. CARLSON, J. GROB and E. WELTY 1993: Forestry in the 21st century. The biotechnology of somatic embryogenesis. Biotechnology **11**, 454–459.

HALOS S.C. and N.E. GO 1993: Micropropagation of *Pinus caribaea* Morelet. Plant Cell Tissue Organ Cult. **32**, 47–53.

HORGAN K and J. AITKEN 1981: Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. Physiol. Plant **53**: 170–175.

MURASHIGE T. and F. SKOOG 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473–497.

PATEL K.R., H.R. KIM and T.A. THORPE 1986: Plantlet formation in pitch pine (*Pinus rigida* Mill) by tissue culture methods. For. Ecol. Mgt. **15**, 147–160.

PULIDO C.M, I.S. HARRY and T.A. THORPE 1992: Optimization of bud induction in cotyledonary explants of *Pinus canariensis*. Plant Cell Tissue Organ Cult. **29**: 247–255.

QUOIRIN M. and P. LEPOIVRE 1977: Etudes de milieux adaptes aux cultures in vitro de Prunus. Acta Hort. **78**, 437–442.

SCHENK R.U. and A.C. HILDEBRANDT 1972: Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. **50**, 199–204.

SOMMER H.E., C.H. BROWN and P. KORMANIK 1975: Differentiation of plantlets on long leaf pine (*Pinus palustris*) Mill tissue cultured in vitro. Bot. Gaz. **136**: 196–200.

STOJICIC D., S. BUDIMIR and L. CULAFIC 1999: Micropropagation of *Pinus heldreichii*. Plant Cell Tissue Organ Cult. **59**, 147–150.

TUSKAN G.A., W.A. SARGENT, and J.A.WALLA 1990: Influence of plant growth regulators, basal media and carbohydrate levels on the in vitro development of *Pinus ponderosa* (Dougl. ex law) cotyledon explants. Plant Cell Tissue Organ Cult. **20**: 47–52.

Eingereicht: 15.4.2000/9.8.2000

Anschrift der Verfasser: *Dr. D. Nandwani, (corresponding author) S. Kumaria und P. Tandon, Plant Biotechnology Laboratory, Agric. Exp. Station, College of Marshall Islands, PO Box 1258, Majuro, MH 96960, Marshall Islands)