Chapter V

*In vitro* culture from axillary bud of *Taxus wallichiana* Zucc.
Introduction:

*Taxus wallichiana* Zucc. is a small or medium-sized evergreen gymnosperm tree with its height ranging from 9-20 m. It belongs to the family Taxaceae. The stem is short, thick (7 m. or more in girth), and is covered by a reddish brown bark that peels off easily. It bears spreading branches, which are surrounded by scales at the base. The shoots bear green foliage leaves which are arranged in a fairly close spiral but becomes spread in two ranks by the twisting and curving of the petioles. The lamina is asymmetrical at the base and narrows down into a petiole having decurrent base, which is persistent in nature. The lamina is entire, dark green, glossy with slightly recurved margin and pointed horny tip.

The tree is strictly dioecious. The young male and female trees are morphologically alike and cannot be distinguished till the time they bear reproductive structures. The male cones occur singly as stalked globose heads in the axil of the foliage leaves on the undersides of branchlets of the preceding year and are more numerous in the distal region of certain branchlets. The cone axis bears at its base few decussate symmetrical perisporangiate microsporophylls, which are arranged spirally.

The female reproductive structures in *Taxus wallichiana* are not organised into a typical cone. On a female tree, an axillary shoot bud develops on a branch, which is protected by a number of scales.

*Taxus* is distributed in Europe, North and South America, Philippines, Celebes, Algeria, Morocco and India. It comprises ten species of which *Taxus*
wallichiana is the common yew. In India *Taxus wallichiana* grows in the shady and humid tracts above of 1800 m. It is distributed all along the Himalayas from Ladakh eastward to Khasi and Jaintia Hills, Naga Hills and Manipur. This plant is distributed at 1700-3400m altitude, along west to east from Afganistan to north eastern Himalayan states in India (Rikhari et al., 2000).

Once upon a time, the plant used to be found in abundance in Indian hills of Darjeeling and Western Sikkim at altitudes ranging between 2286-2590 m. before large scale deforestation wrecked havoc on the regions biodiversity. Today this plant is found only in certain pockets.

Taxol (paclitaxel), a secondary metabolite of the Taxus species, is a known anticancer drug. This anticancer chemotherapeutic agent has a unique mechanism of action for stabilizing microtubules against depolymerization and shows significant antineoplastic properties (Huang et al., 1999). The importance of taxol as a powerful anticancer drug has resulted in tremendous interest in this phytochemical compound.

Huang et al., (1999) reported that a gene of *Taxus baccata* which encodes the enzyme taxadiene synthase (cyclase). Taxol, a complex diterpenoid, has been called by the National Cancer Institute as "the best anticancer agent" developed in the last 15 years. It is found in the bark, needles, roots and other parts of the tree (Wani et al., 1971, Vidensek et al., 1990). Taxol is highly effective in clinical trials for treatment of many cancers such as ovarian cancer, breast cancer, lungs cancer, skin cancer (melanoma) and colon cancer (Rowinsky et al., 1990). Excessive harvesting to meet pharmaceutical requirements for taxol has caused severe threat to the survival
of the species due to its slow growing nature, lengthy seed dormancy (1.5 to 2 years) and poor seed germination. Most of the fruits are eaten by birds and are difficult to collect. Furthermore germination of mature seeds required a series of treatments. Alternative temperature and cold stratification for 8.5 months produced a 50% germination rate (Chien et al., 1994). Vegetative propagation then is an alternative (Sheu, 1985), however, strong topophysis of lateral shoots limited its application for the production of planting stocks. Further the species is unlikely to reproduce in cleared forest areas or under large canopy gaps as young plants need deep shade (Rikhari, 2000). The propagation of Taxus species from stem cutting is relatively easy but the rate of survivability is very poor. Therefore, to ensure the long term survival of T. wallichiana as a species, it is necessary to explore alternative approaches for propagation of T. wallichiana and the production of taxol.

There have been a number of reports of callus formation from several species of Taxus. Bark (Gibson et al., 1993), Pollen (Tulecke, 1959), female gametophyte tissue (Zekteler et al., 1970, Rohr, 1982) and hypocotyls (Zhiri et al., 1994) have been utilized for callus culture of different species of Taxus. However, there have been almost no reports of plantlet regeneration from callus in Taxus either through somatic embryogenesis or indirect organogenesis. Similarly in vitro propagation through axillary bud multiplication has the potential to be an important tool for obtaining efficient true-to-type vegetative propagation of Taxus.

Hence the present study has been undertaken to investigate the conditions for in vitro regeneration of T. wallichiana aiming to develop a
regeneration procedure that could be useful for mass production of *T. wallichiana* in vitro.

I) *In vitro* regeneration through axillary bud multiplication of *Taxus wallichiana*

i) Materials and methods:

a) Plant material and explant preparation:

The young shoot buds (both terminal and axillary) were surface cleaned by treatment with 5% savlon solution for 8 minutes, sterilized in 0.1% (w/v) aqueous mercuric chloride solution for 9 minutes and rinsed in sterile distilled water (5X10 minutes).

b) Culture medium and conditions:

The shoot bud explants were transferred to 20 ml modified Murashige and Skoog medium (MMS) medium (300 mg/l myo-inositol, 5% sucrose, 0.7% agar, pH 5.8) supplemented with benzylaminopurine (BAP), Adenine sulphate (AdS), indole propionic acid (2 iPA), thidiazuron (TDZ), α-naphthaleneacetic acid (NAA) and Polyvinyl pyrollidone (PVP), either individually or in combination, as well as to Murashige and Skoog (MS) (1962) and Gamborg *et al.*, (B5) (1968) medium. Once the optimum phytohormone concentration had been established for shoot bud multiplication, they were maintained on MMS medium supplemented with 1.0 mg/l BAP, 40 mg/l AdS and 0.5 mg/l NAA over 10-12 passages (15 days each). The medium pH had been adjusted to 5.8 prior to adding 0.7% agar (w/v, Qualigens) and was autoclaved at 121°C for 15 minutes. The cultures were incubated at 24±1°C under 16 hours daily illumination with fluorescent light (12000 lux). The medium was dispensed into
25X150 mm culture tubes containing 20 ml medium or 100 ml wide mouth conical flasks containing 50 ml medium each and cotton plugs were used to close the culture tubes.

c) Shoot multiplication:

The explants were cultured in MMS, MS and B₅ medium without phytohormones as well as with BAP, AdS, 2 iPA, NAA, Thidiazuron and PVP. At first the shoot bud explants were cultured in PVP (2 mg/l) and BAP (0.5 mg/l) for 21 days, after that these were subcultured in other growth regulator containing medium. Initially the explants were subcultured every one week for two subculture and then 15 days interval. The PVP was continuously added to the shoot bud multiplication medium.

d) Rooting of shoots:

Separated shoots (4 cm long) were transferred to MMS, MS and B₅ medium without phytohormones as well with NAA, IBA and thiamin HCl either singly or in combination for rooting.

e) Acclimatization:

The rooted plants were carefully removed after 4 weeks from the culture tube or flasks, the roots were washed thoroughly to remove the adhering medium. Plants were transferred to earthen pots containing a mixture of autoclaved soil:sand:compost (1:1:1) and they were immediately covered with transparent polybag. The pots were kept at 22-24°C under 16 hours photoperiod of a high light intensity (12000 lux). The plants were acclimatized by trimming the corners of the poly bags under culture room conditions.
ii) Results:

Shoot bud Multiplication:

MS and B5 medium supplemented with different phytohormones (either singly or in combinations) in varying concentrations was found to be unsuitable for the induction of multiple shoot formation. However, in MMS medium containing different cytokinin and auxins the inoculated shoot bud explants produced shoot primordia varying in number and length at all the concentrations (Table 15).

Browning of explants was observed within a week of inoculation. The explants released excessive phenolic compounds into the culture medium from their cut end and eventually died within a period of 4 weeks. Therefore, initially the shoot bud explants (Fig 17A) were preconditioned on PVP (2 mg/l) and BAP (0.5 mg/l) containing MMS medium for three weeks. Then they were subcultured on MMS medium supplemented with several phytohormones (either singly or in combinations) (Figs 17B, C & D).

Of all the cytokinin tested for shoot regeneration, BAP gave better response than TDZ and 2 iPA. The use of BAP in combination with AdS and NAA resulted in more shoots than any other treatment (Table 15).
Table 15. Effects of BAP, AdS and NAA on axillary bud proliferation in *Taxus wallichiana* after 20 weeks incubation. Results are the mean of 6 replicates±SE.

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<th>Growth regulators (mg/l)</th>
<th>Mean number of shoots</th>
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**Rooting of *Taxus wallichiana***:

Microshoots did not develop roots in MS and B5 medium. For the induction of roots the microshoots were initially cultured in MMS medium augmented with 5 mg/l NAA for 15 days. After that these were subcultured in MMS containing 0.5 mg/l NAA, 1 mg/l IBA and 800 mg/l thiamin HCl for a period of 4 weeks. After the first 15 days of culture a small amount callus was formed at the base of microshoots. After 4 weeks of subculture in MMS
containing 0.5 mg/l NAA, 1 mg/l IBA and 800 mg/l thiamin HC1 a large number of roots sprouted (Fig 17E) from the callus that developed at the base of the microshoots in the first stage of the culture. If the shoots were cultured in MMS fortified with 0.5 mg/l NAA, 1 mg/l IBA and 800 mg/l thiamin HC1 from the beginning, they formed neither callus nor roots. When the concentrations of growth regulators were increased or decreased in the medium the number roots as well as their length decreased.

Acclimatization:

We failed to acclimatize the plantlets in the field. This may be due to harsh climatic condition prevailing in this region. Further attempts to acclimatize the plantlets in their natural growing climates may be undertaken in future.

In vitro regeneration through Callus culture:

i) Materials and methods:

Explant preparation:

The procedure of surface sterilization of the shoot buds, leaves and internodes explants of _T. wallichiana_ was the same as that of the procedure adopted during shoot bud multiplication.

Culture medium and conditions:

The explants were initially placed on B5 medium augmented with 2 mg/l PVP for 15 days. They were then cultured in the B5 medium supplemented with different combinations of growth regulators such as 6-benzyl amino purine (BAP), kinetin, 2,4-dichlorophenoxyacetic acid (2,4 D), α-naphthalene acetic
acid (NAA), indole butyric acid (IBA), L-glutamine, casein hydrolysate (CH), activated charcoal, Polyvinyl pyrrolidone (PVP) and ascorbic acid. The medium pH had been adjusted to 5.8 before it was autoclaved at 121°C for 15 minutes. Cultures were grown at culture room conditions as previously described.

ii) Results:

Initially the explants (shoot buds, leaf or internode) were preconditioned on hormone free PVP (2 mg/l) containing B5 medium for three weeks. Then they were subcultured on B5 medium supplemented with several phytohormones for callus initiation and propagation.

Callus formation from shoot bud, leaf and internode explants was observed about 7 weeks after the initiation of the culture. Shoot bud explants was superior to leaf and internode explants for callus initiation. Addition of 1 gm/l activated charcoal to the medium promoted callus induction in all the explant types. Callus formation was observed from the cut ends and the epidermal surfaces of the young leaves and the cut ends of shoot buds and both the cut surfaces of young stem as protuberances of the central cylindrical zone. Shoot bud derived callus grew comparatively more rapidly (5.2 gm in 5 months) than leaf derived (2.3 gm in 5 months) and internode derived (1.1 gm in 5 months) callus. B5 medium supplemented with 0.5 mg/l BAP, 0.2 mg/l NAA and 1 gm/l activated charcoal proved to be the most suitable for the establishment and growth of the callus (Fig 17F).

Attempts to induce shoot regeneration in the callus with various auxins and cytokinin combinations in varying concentrations were unsuccessful.
In vitro culture from axillary bud of Taxus wallichiana

Fig. 17A. Axillary shoot bud on MMS+PVP (2 mg/l)+BAP (0.5 mg/l).

Fig. 17B & C. Development of shoot buds from the explant cultured on MMS+BAP (0.5 mg/l)+AdS (10 mg/l)+NAA (0.5 mg/l)

Fig. 17D. Shoot bud proliferation on MMS+BAP (0.5 mg/l)+AdS (10 mg/l)+ NAA (0.5 mg/l) after 20 weeks of culture.

Fig. 17E. Induction of roots from in vitro raised shoot.

Fig. 17F. Axillary shoot bud derived callus on B5+BAP (0.5 mg/l)+NAA (0.2 mg/l)+activated charcoal (1 gm/l).
Discussion:

The importance of tissue culture approach for clonal propagation of woody plant including conifers has already been discussed by Patel and Thorpe (1993). However, coniferous species are still considered difficult to propagate. In vitro propagation methods are not established for most of the Taxus species. These include somatic embryogenesis, organogenesis or shoot bud multiplication.

We were able to induce callus from bud, leaf and internode explants in B5 medium augmented with BAP, NAA and activated charcoal although modified MS medium has been reported to be highly effective in terms of Taxus baccata callus induction and growth (Mihaljevic et.al., 2002). However, we failed to achieve plantlet regeneration from callus.

Although we failed to regenerate plantlets from callus, our attempts to regenerate plantlets through multiple shoot formation was successful. The maximum number of shoot buds was initiated in MMS medium in the presence of BAP and NAA along with other additives such as adenine sulphate. The addition of adenine sulphate showed a synergistic effect on shoot bud induction. This promotive effect of adenine sulphate has already been reported in several other plants (Kaur et.al., 1992; Kaur et.al., 1998; Eeswara et al., 1998; Maity et.al., 2001).

Toxic substances such as phenolic compounds which accumulate close to the explants and inhibit growth and development were removed efficiently by placing the explants on MMS medium with 2% PVP for first 15 days of culture.
The results in shoot regeneration in this species are similar to those obtained for several other woody species where a low concentration of auxin can promote growth of axillary buds in combination with cytokinin (Gavidin et.al., 1996, Anand et.al., 2002; Nunes et.al., 2002).

Thiadiazurone, a substituted phenylurea is mainly used as a cotton defoliant and has been shown to exhibit a strong cytokinin like activity. TDZ has been claimed to be as or more effective than BAP for adventitious shoot regeneration from leaves of several woody species (Chevreau et al., 1988; Fasolo et al., 1989; Swartz, 1988). TDZ in combination with NAA facilitate efficient multiple shoot regeneration in flax (Bretagne et al., 1994). The observation we made while working with woody Taxus species, however, is different. Although TDZ in our case promoted shoot proliferation, but it was not as effective as BAP.

Concentration of 2-3% sucrose was found to be the best for induction for most Coniferous species (Thorpe and Patel, 1984), in Norway spruce 1% sucrose was found to be optimal (von Arnold, 1987). 3% sucrose was reported to be best for multiple shoot formation from zygotic embryos in T. brevifolia (Chee 1995). In the present study, however, a higher concentration of sucrose (5%) was found to evoke the best response.

We have made the following observation from our experiment with regard to the rooting of the microshoots of Taxus species.

- Microshoots form roots with difficulty.
• Root formation can only be induced in two stages
  o In stage one microshoots need to be cultured in MMS supplemented with 5 mg/l NAA for 15 days.
  o In stage two the cultured shoots need to be subcultured for 4 weeks in MMS fortified with NAA, IBA and thiamin HCl.
• Thiamin HCl which was found to stimulate adventitious rooting of Taxus species (Chee 1995a) has played a definitive role in the formation of Taxus roots in vitro.

This protocol for in vitro propagation of Taxus avoided problems such as genetic instability and chromosomal abnormalities as it bypassed the callus formation.
Summary:

The effects of 6-benzyl amino purine (BAP), kinetin (K), 2,4-dichlorophenoxyacetic acid (2,4 D), α-naphthalene acetic acid (NAA), indole butyric acid (IBA), L-glutamine, casein hydrolysate (CH), activated charcoal, Polyvinyl pyrrolidone (PVP) and ascorbic acid were tested in B5 medium with respect to callus initiation and subsequent growth from shoot buds, leaves and internode explants of *T. wallichiana*. Preconditioning of the explants in B5 medium augmented with PVP for 15 days and subsequent addition of 1 gm/l activated charcoal to the phytohormone containing B5 medium is essential for the callus induction of all the explants types. Although *Taxus* callus culture exhibited extremely slow growth rate, shoot bud derived callus grow more rapidly than others. B5 medium supplemented with 0.5 mg/I BAP, 0.5 mg/I NAA and 1 gm/l activated charcoal proved to be the best for callus culture. Attempts to induce plantlet regeneration in the callus via indirect organogenesis and / or somatic embryogenesis were unsuccessful.

Adventitious shoots were directly formed on the nodal segments without callus formation, on MMS medium (300 mg/I myo-inositol, 5% sucrose, 0.7% agar, pH 5.8). Preconditioning of the explants on MMS medium supplemented with PVP (2 mg/I) and BAP (0.5 mg/I) for 21 days and subsequent culture on MMS medium containing BAP (0.5 mg/I), AdS (10 mg/I), NAA (0.5 mg/I) and PVP (1 mg/I) facilitated axillary bud initiation and proliferation.

Rooting of microshoots was achieved in two stages. First the microshoots cultured on MMS medium supplemented with 5mg/I NAA for 15 days produced a small amount of callus at the base. Thereafter, in the second
stage, the cultured shoots with basal callus were transferred to MMS medium containing 0.5mg/l NAA, 1mg/l IBA and 800mg/l thiamin HCl producing a large number of roots from the callus. It is worth mentioning that if the microshoots were cultured exclusively in MMS fortified with 0.5mg/INAA, 1mg/l IBA and 800mg/l thiamin HCl from the beginning, they formed neither callus nor roots.