Evaluation and Selection of *Taxus baccata* L. Clones According to their Root Growth Capacity as a Potential Source of Enzymes for Taxol Biosynthesis

By D. EWALD, T. STAUBER and R. ZOCHER*

Federal Research Centre for Foresty and Forest Products, Institute for Forest Genetics and Forest Tree Breeding, Eberswalder Chaussee 3, D-15377 Waldsieversdorf, Germany

(Received 18th June 2001)

Dedicated to Prof. N. KOHLSTOCK on the occasion of his 70th birthday

Abstract

For purposes of gene conservation cuttings of several clones of the rare and in Germany endangered species *Taxus baccata* L. were harvested in different locations of the country. They were treated with a rooting paste to form roots. By measuring their capacity to form adventitious roots (after seven months) as well as the root length and the number of roots per cutting, it was possible to identify and select those clones which showed an extraordinary capacity for root production and growth using a statistical selection procedure. Roots contained taxol and taxol-synthesising enzymes. The amount of taxol in these newly formed roots was enhanced by treatments with methyljasmonate and epibrassinolide. The activity of the biosynthetic enzyme 10-DAB-acetyltransferase was stimulated after a treatment with 50 µM and 100 µM methyljasmonate. Selection of yew clones characterised by an enhanced root formation and growth is a useful principle to get plant material for studies of taxol biosynthesis.

Key words: yew, root growth, taxol biosynthesis.

Introduction

In some areas of their natural habitats *Taxus spec.* (yew) may be considered as an endangered species. The medical importance of *Taxus baccata* L. is well known as a source of paclitaxel = taxol (SUFFNESS, 1995), which was first isolated from stem bark of of *T. brevifolia* NUTT. This compound showed action against ovarian and breast cancer. The main source of taxol for medical use has been the bark (0.014% dry wt) of *T. brevifolia* NUTT. (STULL, 1992). However, the needles, stem and bark of several *Taxus* species, including *T. baccata* L., have been reported to contain taxol and 10-deacetylbaccatin III (WITHERUP et al., 1990). Also the enzymatic formation of taxol in growing root tips of *T. baccata* L. was shown by ZOCHER et al. (1996).

A programme directed toward gene conservation of *Taxus baccata* L. by cutting propagation has been established at different locations in Germany. A part of results was presented by SCHNECK (1996). Cuttings from *Taxus* trees different in age were harvested and rooting experiments were carried out to establish field trials for ex situ conservation. SCHNECK (1996) investigated the relations of age and sex concerning the rooting behaviour from yew trees of Mecklenburg-Vorpommern. Evaluation of rooting data (e.g. rooting percentage, root length) from cuttings of yew trees derived from different areas in Germany, as shown in this paper, can be used to select clones from each location that have a high rooting potential and that also have roots which grow fast. These clones can generate sufficient root biomass for use in taxol biosynthesis studies. This study was designed to identify such clones and evaluate the production of taxol and the influence of elicitors on taxol formation in rooted cuttings.

Material and Methods

Plant material

Branches from yew trees were harvested in different parts of Germany. Fifty-four yew trees of different age (40–350 years) were selected in Mecklenburg-Vorpommern. Branches were harvested in August 1994. Preliminary experiments with this plant material have evaluated their rooting ability (SCHNECK, 1996) and their genetic characterisation by isozyme patterns (HERTEL, 1996 a, b). Branches from 121 younger trees of *T. baccata* (most of them approximately 55 years old) from a smaller stand in Brandenburg (near Chorin) were harvested in February 1996. Autochthonous plant material from 80 trees in Sachsen-Anhalt was harvested in February 1997. Cuttings (10–12 cm) were taken from the terminal portions of each branch containing one-year-old wood.

Rooting experiments

The methodology for the rooting experiments was based on previous investigations (EWALD and STAUBER, 1994). The rooting experiments were conducted in different years, but all were 7 months in duration. The number of cuttings and repetitions are as follows: A) Mecklenburg-Vorpommern – 54 trees were sampled with 96 cuttings per tree placed in 4 replications; B) Brandenburg (Chorin) – 121 trees were sampled with 48 cuttings per tree placed in 2 replications; and C) Sachsen-Anhalt – 80 trees were sampled with 48 cuttings per tree placed in 2 replications. The experiment with Mecklenburg-Vorpommern trees was conducted from August to March (7 months). Experiments with Brandenburg and Sachsen-Anhalt trees were conducted from February until September. Cuttings (24) from each tree were present in each replication in the various experiments. The chosen size of the cuttings was restricted to 10–12 cm to obtain standardised conditions.

Each cutting was treated with a rooting paste containing 3-indolyl butyric acid (2 g l^{-1}) and placed in sand in small plastic „mini-greenhouses“ (50 x 20 x 20 cm) within a greenhouse for rooting. The greenhouse was kept cool (5 °C) during wintertime. To prevent fungi attack, the cuttings were sprayed with a solution of 0.2% EUPAREN (50% dichlorfluanil) at the beginning of the experiment. After seven months, rooting percentage was determined, root number was counted and root length (mm or cm) was measured for every rooted cutting. For calculations the sum of the lengths of all roots per cutting was used.

*Institute for Biochemistry and Molecular Biology, Technical University Berlin, Franklinstrasse 29, D-10587 Berlin, Germany

Mathematical and Statistical procedures

The method of GUPTA (1956, 1965) was chosen to identify the best stecklings (clone) within a group of donor trees with a definite significance (95%), also called subset selection method. Calculations were carried out for total root length as well as for the root number. Because the amount of rooted cuttings (rooting percentage) is an important factor for biomass at harvest, the rooting percentage of each clone was included in the calculations as a factor to weigh the results. The average root length was therefore multiplied with the rooting rate (= rooting percentage/100) of each clone ($0 \leq$ rooting rate ≤ 1).

The following selection procedure according to GUPTA (1956, 1965) was used:

$1 - \alpha$	probability of selecting the best clone
n	observations per mean (clone)
a	count of means to select
$\bar{x}_{\max}$	maximum of a means
$\bar{x}_1, \ldots, \bar{x}_a$	'a' means to select
$s_1^2, \ldots, s_a^2$	'a' variances of means to select
$s^2 = \frac{\sum_{i=1}^{a} s_i^2}{a}$	the estimation of common variance of each means
$t_{a-1(n-1),\, 0.5,1-\alpha}$	one sided (1-α)-quantil of the (a-1) dimensional t-distribution in degrees of freedom a(n-1) and with the coefficient of correlation 0.5 (table 3a, page 259 in: HORN and VOLLANDT, 1995)

We selected all means x_i, beyond a threshold of selection, where:

$$x_i \geq x_{\max} - t_{a-1(n-1),\, 0.5,1-\alpha} \bullet s\sqrt{\frac{2}{n}}$$

Depending on the values for the highest root length a threshold root length for selection was calculated for the clones of each location. Clones with a weighed root length beyond this value were selected. This part included the most outstanding clones concerning the root length and rooting percentage (HORN and VOLLANDT, 1995).

Our aim was to find out by mathematical methods the group of clones in every location (provenance) which includes the best clone with a definite significance (95%), concerning root length. A second aim for the mathematical treatment was that the rooting percentage should be included in the ranking as well. This should demonstrate the ability of each clone to form roots from a practical point of view, to evaluate its root forming capacity in total.

Estimation of taxol content in newly formed roots (fast growing white root parts)

We wished to know if taxol was present in the white and fast growing root parts derived from cuttings. For this reason a part of the experimental material (3 stecklings per donor tree from 40 different mother trees, Mecklenburg-Vorpommern) were used to test for the presence of taxol. After the rooting experiment, plants were therefore transferred to 1–liter containers. Fast growing white root parts which grew out of these containers into a 5 cm layer of wet peat under the containers were harvested from the clones after 3 months. These roots (diameter of approximately 1–2 mm) were collected, washed and taxol content was analysed according to ZOCHER et al. (1996). The amount of taxol was calculated per gram dry weight (DW) of root mass. The average amount of taxol and the standard deviation was calculated.

Stimulation of taxol content in fast growing root parts

Rooted cuttings of one clone were used to study the influence of elicitors on taxol formation after the rooting period. Five rooted cuttings per variant were grown in an aerated mineral solution (DERDULLA et al., 1997). The experiment was a treatment of the roots in the mineral solution with either 50 µM methyljasmonate or 100 nM 24-epibrassinolide for one week compared with a control. Roots were harvested after that period and the content of taxol was analysed.

Experiments to test the influence of methyljasmonate on the activity of 10-DAB-acetyltransferase

Treatment of the roots:

Twenty rooted *Taxus* cuttings per variant (clone MV 38) were cultured in an aerated hydroponic system (6 liter volume). Methyljasmonate (50 µM and 100 µM) was suspended in 5 ml 30% ethanol and added to the nutrient solution for 72 hours. The control variant was treated with 30% ethanol exclusively. After the treatment white root parts were harvested and stored at –80°C immediately.

10-DAB-acetyltransferase detection:

Two gram of frozen *Taxus* root material was ground in a mortar in the presence of liquid nitrogen to a fine powder. Ten ml of 50 mM MOPS-buffer (pH 7.4) was added and stirred for 1 hour on ice. After centrifugation 1 ml of the supernatant was desalted using a PD 10-column (Pharmacia) and afterwards used for the enzyme test. An aliquot of the enzyme solution (100 µl) was incubated with 0.5 µCi 14-C-acetylcoenzyme A and 200 µM 10-deacetylbaccatin-III for 15 minutes at 30°C. The mixture was then diluted with 2 ml distilled water and extracted with EtOAc. The organic phase was evaporated and the residue was dissolved in 50 µl EtOAc and separated by thin layer chromatography (silica gel, solvent CHCl3:MeOH = 95:5). Baccatin-III (Sigma) was used as a marker. The radioactivity was determined by radioscanning (BERTHOLD).

Results and Discussion

Rooting

Because yew is a rare tree species in Germany, stands of yew trees are the exception. The trees are in most cases single trees or little groups situated in larger areas (progenies) and their origin is not known. Nevertheless a genetic analysis of yew trees in Mecklenburg-Vorpommern showed that they are different from other locations (HERTEL and KOHLSTOCK, 1996). That is why the selection of single trees was the aim of the investigation and not the comparison of the material from different locations.

The parameters for the highest and lowest root length of the different clones from different locations and the variation of rooting percentage (highest versus lowest) is shown in *Table 1.* The highest and lowest rooting percentage of clones from each location reached from 4% to 100% (Sachsen-Anhalt), 5% to 92% (Mecklenburg-Vorpommern) and 10% to 100% (Chorin).

Based on the results of SCHNECK (1996) with identical plant material from one of the locations (Mecklenburg-Vorpommern) it became obvious that 71.2% of the variance for rooting percentage resulted from clone effects. As shown by the same author root growth did not depend clearly from age and sex of the donor trees. These conclusions were the basis for our experiments and calculations to select clones with a maximum root growth.

Rooting percentage, the total root length and the root number, as the chosen traits, provide sufficient information about the general rooting capacity of clones.

Table 1. – Selection of the clone with the highest total average root length (weighed by average rooting percentage[=multiplied with rooting percentage/100]). The best clone of each provenance is included in the chosen clones with a probability of 95%.

		highest clonal average root length in mm	root length, threshold for selection (mm)	number of selected clones	lowest clonal average root length in mm (minimal)	average variance	rooting percentage of clones (%) within the provenance	
provenance	no. of clones tested						lowest	highest
Chorin	121	588.39	498.5	4	9.37	16416.07	10	100
Sachsen Anhalt	78	338.46	273	4	0.21	5758.14	4	100
Mecklenburg Vorpommern	50	109.34	96.56	2	1.36	945.95	5	92

The selected top group of clones contained 10 clones out of 255 clones tested in total (*Table 2*). This mathematical procedure allowed us to find out the group of clones in every location (provenance) which included the best clone with a definite significance (95%) concerning root length. The information about rooting behaviour in general was included by using the rooting percentage as a weighing factor (rooting rate) in the ranking as well. This demonstrated the ability of each clone to form roots from a practical point of view.

Table 2. – Ten yew clones of different provenances with the best root growth within the provenance were selected by the procedure of GUPTA (co - Chorin, st - Sachsen Anhalt, mv - Mecklenburg/Vorpommern).

provenance/clone	average root number	average total root length (mm)	rooting percentage (%)
co_1_12	10.39	588.39	100
co_1_18	15.67	594.96	96
co_1_29	12.23	536.85	96
co_2_60	10.89	559.70	92
st_16d	8.96	331.43	96
st_20a	7.26	311.91	96
st_25/C	6.96	313.43	96
st_27	7.95	369.23	92
mv_24	3.81	119.28	92
mv_40	4.77	122.80	82

The material and experimental conditions varied within the experiments.

The time of choice to start such investigations was August. The autumn period was preferred because the average daily temperature was lower, which was better for the root induction process. A higher temperature, as during spring, forced the callus formation at the basal end of cuttings which were treated with auxins (EWALD, personal observation). In two experiments the cuttings were provided in February. Calculating the data a variation in the root forming capability depending on the genotype was observed.

Because the experimental conditions were not identical for all three rooting experiments, it would be desirable to test all plant material in one single experiment so as to facilitate the final ranking of clones. This would allow us to draw a general conclusion for all 255 clones involved in this consideration based on an identical rooting treatment applied to all clones. Nevertheless the chosen method of GUPTA allowed the selection of suitable donor trees within a broad variety of trees providing stecklings which form sufficient amounts of fast growing root material.

Taxol content in growing root parts and its stimulation

A formation of taxol in roots was already shown by WICKREMESINHE and ARTECA (1994) and DERDULLA et al. (1997).

In our experiment the taxol content in the white and fast growing root parts derived from cuttings showed an average value of 109.8 µg/g ± 77.2 µg/g DW in the forty samples. Thus it is evident that these root parts or root tips (Mecklenburg-Vorpommern) contained taxol.

The aim was to select clones for a system where the growing roots can be used to extract several enzymes of the taxol biosynthesis. Taxol biosynthesis was already detected in root tips of nonselected *Taxus baccata* cuttings (ZOCHER et al., 1996).

Thus it was assumed that an increase in the taxol content by elicitors shows the presence of enzyme activity as well as the possibility to stimulate the enzymes in these plant parts (roots). The results of the elicitor treatment confirmed this assumption. The amount of taxol in the fast growing white root parts could be enhanced by adding 50 µM methyljasmonate or 100 nM epibrassinolide to the hydroponic system. For the control, in which only the solvent was added, 23 µg/g FW of taxol, was present in roots whereas methyljasmonate or epibrassinolide treated roots contained 56.9 or 56.96 µg/g FW respectively. Thus the amount of taxol was approximately doubled.

Stimulation of 10-DAB-acetyltransferase activity

For experimental purposes (sufficient amount of material) a clone with an average rooting behaviour (MV 38 – rank 28 from 54 trees from Mecklenburg-Vorpommern according to the chosen selection method) was chosen to test the influence of an elicitor treatment. The activity of the enzyme after a treatment of methyljasmonate for three days was enhanced substantially as shown in *Fig. 1.* The activity after 50 µM methyljasmonate was 3.5 fold whereas after 100 µM it was 2.9 fold of the control. Experiments with roots of other clones (e.g. MV 31 – rank 5 of 54 trees from MV) confirmed the stimulating effect of methyljasmonate (ZOCHER, personal communication).

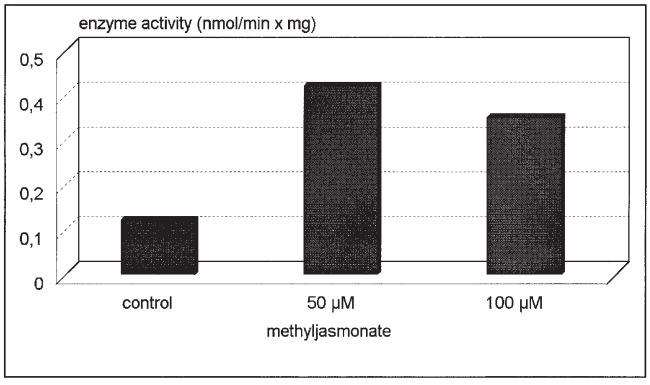

Figure 1. – Stimulation of the 10-DAB-acetyltransferase activity of *Taxus* roots in hydroponic culture after a 72 hour treatment with methyljasmonate.

Concluding from these results it is possible to say that the biosynthesis of taxol was enhanced in these parts of growing roots. Similar stimulating effects of methyljasmonate were described for cell cultures of *Taxus* (YUKIMUNE et al., 1996). Thus selected *Taxus* clones with roots showing a high growth rate and therefore an extraordinary amount of white root areas are the material of choice for the extraction of taxol synthesising enzymes. They are a suitable source of plant material for experiments, because active cambium is not available during the whole year.

Concluding from these results, it was possible to select yew-clones among a variety of clones of different origin with a high rooting ability and root growth which can be used in ongoing research work for investigations concerning taxol biosynthesis on all levels.

Acknowledgement

The help of the following colleagues to provide the plant material is kindly acknowledged: FD D. KÖHLER (Mecklenburg-Vorpommern), Prof. N. KOHLSTOCK and CHRISTINA BENDIX (Sachsen-Anhalt).

References

DERDULLA; H.-J., GLUND, K., HÄUPKE, K., EWALD, D., RÖNSCH, R., ZOCHER, R., HACKER, C. and WECKWERTH, W.: Verfahren zur Gewinnung von Taxanen. Patent; DE 196 23 338 C1; (1997). — EWALD, D. and STAUBER, T.: Untersuchungen zur vegetativen Vermehrung von Eiben. Beiträge für Forstwirtschaft und Landschaftsökologie **28**, 2, 70–72 (1994). — GUPTA, S. S.: On a decision rule for a problem in ranking means. Ph. D. thesis (Mimeo Ser. No. 150). Institute of Statistics, University of North Carolina, Chapel Hill (1956). — GUPTA, S. S.: On some multiple decision (selection and ranking) rules. Technome trics **7**, 225–245 (1965). — HERTEL, H.: Vererbung von Isoenzymmarkern bei Eibe (*Taxus baccata* L.). Silvae Genetica **45**, 5–6, 284–290 (1996a). — HERTEL, H. and KOHLSTOCK, N.: Genetische Variation und geographische Struktur von Eibenvorkommen (*Taxus baccata* L.) in Mecklenburg-Vorpommern. Silvae Genetica **45**, 5–6, 290–294 (1996b). — HORN, M. and VOLLANDT, R.: Auswahl einer Teilmenge, die die beste Population enthält. In: Biometrie – Multiple Tests und Auswahlverfahren, HORN and VOLLANDT (eds.), Gustav Fischer Verlag, Stuttgart, Jena, 181–188 (1995). — SCHNECK, V.: Untersuchungen zur Klonabhängigkeit der Bewurzelungsfähigkeit und der Qualität der Wurzelbildung bei der Stecklingsvermehrung von 40–350jährigen Auslesebäumen der Eibe (*Taxus baccata* L.). Silvae Genetica **45**, 5–6, 246–249 (1996). — STULL, D. P.: Current taxol production from yew bark and future production strategies. Second National Cancer Institute Workshop on Taxol and *Taxus*: September 23–24, 1992. Alexandria, Virginia (Abstract) (1992). — SUFFNESS, M. (ed.): Taxol: science and application. Boca Raton, USA; CRC Press, Inc. 426 pp. (1995). — WICKREMESINHE, R. M. E. and ARTECA, R. N.: Roots of hydroponically grown *Taxus* plants as a source of taxol and related taxanes. Plant Science **101**, 125–135 (1994). — WITHERUP, K. M., LOOK, S., STASKO, M. W., GHIORZI, T. J., MUSCHIK, G. M. and CRAGG, G. M.: *Taxus* spp. needles contain amounts of taxol, comparable to the bark of *Taxus* brevifolia: analysis and isolation. J. Nat. Prod. **53**: 1249–1255 (1990). — YUKIHITO YUKIMUNE, HOMARE TABATA, YOSUKE HIGASHI and YASUHIRO HARA: Methyljasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. Nature biotechnology (**14**) 1129–1132 (1996). — ZOCHER, R., WECKWERTH, W., HACKER, C., KAMMER, B., HORNBOGEN, T. and EWALD, D.: Biosynthesis of Taxol: Enzymatic acetylation of 10-deacetylbaccatin-III to baccatin-III in crude extracts from roots of *Taxus baccata*. Biochem. and Biophys. Research Communications **229**, 16–20 (1996).

Genetic Variation in Height Growth among Populations of Eastern White Pine (*Pinus strobus* L.) in Ontario

By D. G. JOYCE[1]), P. LU[2]) and R. W. SINCLAIR

Ontario Ministry of Natural Resources, Ontario Forest Research Institute, 1235 Queen Street E., Sault Ste. Marie, ON, Canada P6A 2E5

(Received 11th September 2001)

Abstract

Provenances of eastern white pine (*Pinus strobus* L.) were sampled from natural populations in Ontario and assessed in a 2-year greenhouse study followed by two 5-year short term field tests to reveal the scale and patterns of genetic variation in growth and adaptation at early ages. Results indicate that considerable genetic variation in growth potential exists among white pine populations in Ontario and that appreciable genetic gain can be realized through seed source selection in reforestation programs. Strong age-age correlation between growth traits at different years and the weak genotype-by-provenance interaction suggested high efficiency in provenance selection at early stage. Due to the latitude predominated clinal pattern of geographic variation and the significant reduction in growth potential when seeds are transferred from north to south, it is recommended not to use seed sources 1.5 to 2.0 degrees (latitude) north of a place where regeneration of eastern white pine occurs. Because of the relatively mild test environments at two sites and the short-term nature of this study, further research is required to investigate differences in cold hardiness among provenances before safe south to north seed transfer distances can be determined.

Key words: Pinus strobus, seed source, genetic variation, growth potential, seed transfer.

Introduction

For forest tree species with large natural distributions, such as the eastern white pine (*Pinus strobus* L.), substantial genetic variation in growth among provenances has been reported from range-wide provenance tests (FOWLER and HEIMBURGER, 1969; GARRETT et al., 1973; GENYS, 1987; ABUAKER and ZSUFFA, 1991). While range-wide provenance tests are valuable for revealing large-scale geographic patterns of genetic variation, as well as in identifying potentially superior provenances, results from these tests with eastern white pine are insufficient to satisfy operational forest regeneration needs, such as seed source selection, in Ontario. The difficulty exists mainly because only a few eastern white pine population samples from Ontario were included in these range-wide provenance tests (FOWLER and HEIMBURGER, 1969) and the test sites were not representatives of the environmental conditions in Ontario where most eastern white pine regeneration takes place. The

[1]) Current address: Ontario Ministry of Natural Resources
70 Foster Drive, Suite 400
Sault Ste. Marie, ON, Canada P6A 6V5

[2]) Corresponding author: Email: pengxin.lu@mnr.gov.on.ca