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BIOTECHNOLOGICAL APPROACHES FOR PROPAGATION OF *TAXUS BACCATA* L. - AN ENDANGERED PLANT WITH IMPORTANT ORNAMENTAL AND PHARMACEUTICAL VALUE

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ABSTRACT:

Taxus is propagated by seeds and rooted cuttings though these methods are slow and can not respond to the growing demand of the planting material. The objective of our study was to refine a procedure for *in vitro* shoot culture of *T. baccata* L. Explants were obtained from a mature tree and after disinfection were inoculated on media involving various basal salts and plant growth regulators. WPM nutrient medium supplemented with 6.84μM zeatin exhibited the best initiation of shoot apices with high frequency of axillary bud induction averaged 2-3 buds/explant. WPM lacking growth regulators supported shoot elongation.

KEYWORDS: *in vitro*, shoot culture, axillary bud, mature tree, zeatin

INTRODUCTION

Interest in over exploitation, possibly illegal, of the existing resources of *Taxus* has been intensified with the discovery of Taxol® which is identified as one of the most promising anti-cancer drugs [6]. *Taxus* is propagated by seeds and rooted cuttings though these methods are slow and would not conserve the plant traits people value. *In vitro* culture of *Taxus* has been reported mainly through embryo culture and somatic embryogenesis.

Axillary bud induction from juvenile shoot tips or segments derived from rooted cuttings [5,8], *in vitro*-germinated seedlings [10] or even from up-to 5-year-old plants [13] has also been studied. Meanwhile, using mature plant materials which could be up to 1000-year-old is limited [4,2], attributable to the fact that micropropagation of mature trees is generally more difficult comparing to their juvenile counterparts. Nevertheless, mature trees are often preferred for cloning because one can select trees that have been in the field long enough to have demonstrated their superior value.

Using shoot tips as initial explants results in a problem of elongated single shoot without axillary bud induction [13], which could be overcome by shoot apical decapitation [10].

The objective of our study was, therefore, to refine a procedure for initiation, establishment and proliferation of an in vitro shoot culture from mature explants of *Taxus*.

MATERIALS AND METHODS

Shoot cultures were initiated from shoot apices obtained from a mature tree of *T. baccata*. Explants (shoot apices 1.5-2.5 cm long) were collected during March-April or later in October-November when new vegetative buds usually appear.

Explants were disinfected according standard procedure – washing and five minutes treatment with a 5% (w/v) solution of calcium hypochlorite [$Ca(ClO)_2$] with constant agitation was given. Calcium hypochlorite was washed off three times with sterilized distilled water, 10 minutes each, under aseptic conditions. Explants were then separated into shoot apices and shoot nodal segments, prepared by removing the needles and inoculated on various growing media involving various basal salts – MS [11] or WPM [9] and different combinations of plant growth regulators (PGR) (Table 1). Carbohydrates (sucrose and/or glucose) and 5 g/l agar (in case of solid media), were added to the nutrient media after adjusting the pH to 5.6. At least 20 explants were used per treatment.

In vitro cultures were kept at 22±2°C under 16-h photoperiod (fluorescent tubes OSRAM 40 W, 40 μmol m^{-2} s^{-1} PPED).

RESULTS AND DISCUSSION

Despite the difficulties facing micropropagation of mature trees, our studies were initiated with explants from a single mature tree of *T. baccata*. Total percentage of contamination was recorded ranging from 50% to 90%. This problem was successfully solved by using silver nitrate as disinfection agent [7].

At the beginning, a general screening of various combinations of basal media with different PGR was performed. Irrespective of explant type, all explants cultured on liquid media appeared swollen, formed callus in some cases and almost died shortly after culturing. Shoot apices explants survived on solid media containing either concentration of BAP (8.88 μM or 13.32 μM), even though with no further development. Explants were examined under binocular which revealed the viability of approximately all shoot apices. However, no further development was noticed in any explant even after several subcultures to identical fresh medium. Shoot nodal segments, on the other hand, proved to be improper as initial explants from *T. baccata* as they deteriorated shortly after the first culture. Shoot apices, therefore, have been used as initial explants through the course of the study and nodal segments were excluded.

Using explants from mature trees in the present experiment was the main problem encountered culture initiation and establishment. Most of the plants express cellular totipotency only in the cultures of embryonic explants. In many woody species even propagation by shoot bud proliferation has been successful only with juvenile tissues. Older tissues of these plants have remained recalcitrant. Probably in these species cells lose their competence to respond to the inductive conditions very early during development [3].

Under the condition of our experiment explants of *T. baccata* failed to grow and develop on MS basal medium at full or reduced ionic strength supplemented with any of the hormonal combinations previously mentioned, although MS medium containing BAP has been recommended by several investigators for micropropagation of *Taxus* [12,8,2]. One over-riding cause why MS medium doesn't support good growth of many woody plants is the total ionic strength (100.48mM). The relatively high concentrations of BAP tested in our experiment maybe also contributed to a great extent in the failure of explant initiation and development. Conifer cultures are sensitive to cytokinins, as pointed out by Amos and McCown [1]. At high concentration, auxin inhibits the development of the primordial or axillary buds and induces the formation of callus whilst shoots elongate better on media containing lower concentrations of PGRs [4]. Besides, explants survived only on solid media which could be due to the good physical contact of the explants with the nutrients and preventing them from submersion into a liquid medium. Liquid media, however, have low oxygen content, no gaseous exchange and suppressed transpiration. According to these results, we deduced that using liquid media, reduced MS salts and BAP at high concentrations were improper for culture initiation in *Taxus*.

Explants cultured on media supplemented with IBA in combination with BAP suffer severe wilting especially those grown on media containing the highest concentration of BAP plus IBA. Although, the sole treatment of BAP exhibited appropriate survival rate of the explants, no sprouting was achieved. Apical buds were green and seemed alive under binocular, though without any sign of development.

Having in mind the results from the preliminary experiments we decided in the next step of our study to test the effect of basal medium with low ionic strength supplemented with zeatin instead of BAP. WPM was chosen as a medium with a lower ionic strength (43.15mM). WPM was used either with or without the addition of 6.84 μM zeatin. No development was observed throughout the first three weeks following the culture. Explants grown on zeatin-supplemented WPM were characterized by induction of apical and axillary buds in high frequency with an average of 2-3 buds/explant. On hormone-free WPM, however, lower frequency was noticed with an average of single elongated shoot per explant (Fig. 1.). Although zeatin boosted bud induction, it did not support shoot elongation. The conclusion was to establish and to maintain a propagation culture on induction medium and then to transfer buds to an elongation medium. In the following subculture, therefore, all explants generated from zeatin-containing medium were transferred into a medium lacking growth hormones for shoot elongation and vice versa for bud induction. This alternate culture allowed the elongation of newly developed buds during the previous phase.

In accordance with our findings, Ewald [5] working with *T. baccata*, tested many efficient cytokinins, like BAP, to stimulate bud and shoot development in combination with nutrient media and found that it led to a rapid necrosis of the material. WPM, however, was the most efficient for the growth and vitality of explants. Besides, zeatin could be used for bud induction in *T. baccata* for it was

successful with other conifers like larch, Norway spruce. WPM has been suggested by other authors for micropropagation of various *Taxus* species [10,5].

After the primary proliferation noticed on WPM with zeatin, secondary proliferation was low or even absent in most cases. Developing new media was therefore inevitable for higher secondary proliferation. Single shoots of *Taxus* obtained from primary sprouting were used. WPM nutrient media supplemented with various combinations of PGR (zeatin, BAP, kinetin and TDZ) and casein hydrolysate (CH) were tested.

In our control treatment of 6.84µM zeatin, which proved adequate for primary sprouting, further proliferation was absent and only single elongated shoot was obtained with no axillary bud induction. Soon after elongation, the uppermost new leaves deteriorated and appeared yellowish green. Supplementing WPM with 2.5µM TDZ led to the worst results. Explant growth on this medium was retarded, almost all leaves desiccated and only the apical bud was alive with no development. When the concentration of TDZ was raised to 13.69µM, elongated shoots were attained though with pale-green and sometimes dead explants. Combining zeatin with TDZ resulted in compacted green shoots with new leaves condensed at the top. Unfortunately, secondary sprouting was not possible as we had anticipated.

CONCLUSIONS

- WPM nutrient medium supplemented with 6.84µM zeatin exhibited the best initiation of shoot apices with high frequency of axillary bud induction averaged 2-3 buds/explant. WPM lacking growth regulators supported shoot elongation.
- Secondary sprouting was not possible on media containing WPM supplemented with at casein hydrolysate (CH) and any of the combination between zeatin and TDZ.

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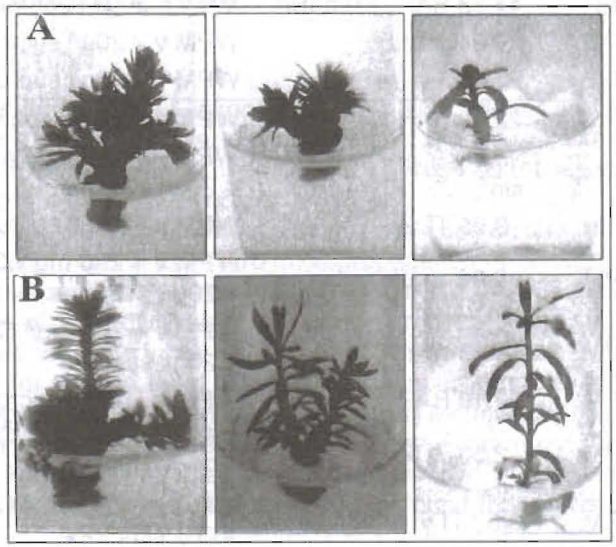

Fig. 1. Induction and elongation of axillary buds of *Taxus baccata*. L.
A. solid WPM + 6.84μM zeatin. **B.** elongation on hormone-free WPM medium.

Table 1.

Basal media and growth regulators used for *Taxus baccata*. L. shoot culture initiation and proliferation .

Stage	Basal medium	Supplements	
		Growth regulator µM	Others [v≡ vitamins, CH≡ casein hydrolysate]
Initiation	¼ MS liquid	8.88 BAP	MS v + 20g/l sucrose + 20g/l glucose
	¼ MS liquid	13.32 BAP	MS v + 20g/l sucrose + 20g/l glucose
	¼ MS	8.88 BAP	MS v + 20g/l sucrose + 20g/l glucose
	¼ MS	13.32 BAP	MS v + 20g/l sucrose + 20g/l glucose
	MS	4.44 BAP	MS v + 30g/l sucrose
	MS	22.19 BAP	MS v + 30g/l sucrose
	MS	44.38 BAP	MS v + 30g/l sucrose
	MS	4.44 BAP + 0.5 IBA	MS v + 30g/l sucrose
	MS	22.19 BAP + 0.5 IBA	MS v + 30g/l sucrose
	MS	44.38 BAP + 0.5 IBA	MS v + 30g/l sucrose
	WPM	6.84 zeatin	WPM v + 20g/l sucrose
	WPM	Hormone-free	WPM v + 20g/l sucrose
Proliferation	WPM	22.19 BAP	WPM v + 20 g/l sucrose
	WPM	3.0 zeatin + 3.0 kin	WPM v + 100 mg/l arginine + 20g/l sucrose
	WPM	0.05 TDZ	WPM v + 20g/l sucrose
	WPM	**6.84 zeatin**	**WPM v + 250 mg/l CH + 20g/l sucrose**
	WPM	Hormone-free	WPM v + 250 mg/l CH + 20g/l sucrose
	WPM	22.19 BAP	WPM v + 20g/l sucrose
	WPM	2.50 TDZ	WPM v + 250 mg/l CH + 20g/l sucrose
	WPM	0.50 TDZ + 6.84 zeatin	WPM v + 250 mg/l CH + 20g/l sucrose
	WPM	13.69 zeatin	WPM v + 250 mg/l CH + 20g/l sucrose