Trees (2011) 25:577–584
DOI 10.1007/s00468-010-0534-6

ORIGINAL PAPER

The role of cytokinins on in vitro shoot production in *Salix tetrasperma* Roxb.: a tree of ecological importance

Md Imran Khan · Naseem Ahmad · Mohammad Anis

Received: 29 July 2010 / Revised: 3 December 2010 / Accepted: 15 December 2010 / Published online: 5 January 2011

Abstract A valuable tropical tree, *Salix tetrasperma* Roxb. commonly known as Indian willow has been investigated for its in vitro regeneration potential using nodal explants obtained from a 30-year-old elite tree. Agar-solidified Woody Plant Medium (WPM) containing different concentrations of Plant Growth Regulators (PGRs) was used in the study. Shoot induction response was best on WPM supplemented with 6-benzyladenine (5.0 μM) where 90% explants responded with an average shoot number (4.40 ± 0.50) and shoot length (0.92 ± 0.04) after 6 weeks of culture. However, multiplication and elongation was best recorded when BA (5.0 μM) treated shoot clusters were transferred to WPM containing BA (1.0 μM) + NAA (0.5 μM) where 18.40 ± 0.92 well-grown healthy shoots with an average shoot length of 5.30 ± 0.43 cm were obtained on completion of 12 weeks culture period. In vitro rooting of shoots was best achieved in half-strength WPM containing 0.5 μM IBA. Well-rooted plantlets were successfully hardened off and acclimatized in plastic cups containing sterile Soilrite. These plantlets were then transferred to pots containing normal garden soil followed by transfer to greenhouse and ultimately to field under full sun.

Keywords Micropropagation · *Salix tetrasperma* Roxb. · Shoot induction · Shoot multiplication · In vitro rooting

Abbreviations

BA	6-Benzylaminopurine
NAA	α Naphthalene acetic acid
WPM	Woody plant medium
PGRs	Plant growth regulators

Introduction

The genus *Salix* commonly known as willow of the family Salicaceae is a deciduous tree or shrub found primarily on moist soils in cold and temperate regions of the northern hemisphere (Anonymous 2003). There are about 400 species of willow. Many willow species are very vigorous growers and are characterized by particular physiological adaptation (Park et al. 2008). The economic importance of the various species is currently increasing and emerging in a wide array of practical application to restore damaged ecosystems including phytoremediation and bioengineering such as the production of biofuels (Perttu and Kowalik 1997; Granel et al. 2002; Vervaeke et al. 2003; Kuzovkina and Quigley 2005). *Salix tetrasperma* Roxb. also known as Indian willow is a valuable tree that could be used as a potential tree for phytoremediation. Besides, the plant is also used as an antipyretic, analgesic, and astringent. The dried and powdered leaves mixed with sugar is given for the treatment of several diseases, such as rheumatism,

Communicated by J. Carlson.

Electronic supplementary material The online version of this article (doi:10.1007/s00468-010-0534-6) contains supplementary material, which is available to authorized users.

M. I. Khan · N. Ahmad · M. Anis
Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh 202 002, India

M. Anis (✉)
Department of Plant Production, College of Food and Agriculture Science, King Saud University, PO Box 2460, Riyadh 11451, Saudi Arabia
e-mail: anism1@rediffmail.com

epilepsy, swellings, piles, venereal diseases, and stones in bladder (Anonymous 2003). The drug 'Aspirin' derived from *Salix* is used as non-steroidal anti-inflammatory drug (NSAIDS). Recently willows have been extensively utilized for production of biofuels because of their fast and rapid growing habit.

Rapid urbanization coupled with multifaceted and ever increasing demands of humankind has taken a toll on the natural population of many forest trees. Regeneration and restoration of these populations in the natural conditions is very low resulting in shrinking forest resource (Giri et al. 2004). In such scenarios, commercialization and exploitation of valuable forest resource needs a serious concern and steps or efforts need to be taken towards conservation and sustainable utilization of important forest trees. Conventional propagation cannot fulfill the need for rapid introduction of newly selected genotypes and in vitro propagation is the ultimate solution for this and several other woody species (Thorpe et al. 1991; Ahuja 1993; Vinocur et al. 2000). In addition, the germplasm of economically significant species may be preserved ex situ in culture collections and gene banks, circumventing the need to continuously take germplasm from natural environments. Furthermore, tissue culture of utilizable species can be used as a direct source of natural products. In vitro propagation from adventitious or axillary buds is a useful technique for mass propagation of many valuable plant species (Faisal and Anis 2002; Ahmad and Anis 2007). There are several reports on in vitro propagation of willows (Bhojwani 1980; Chalupa 1983; Dhir et al. 1984; Bergman et al. 1985; Grönroos et al. 1989; Stoehr et al. 1989; Neuner and Beiderback 1993; Agrawal and Gebhardt 1994; Amo Marco and Lledo 1996; Lyyra et al. 2006; Park et al. 2008) but, so far no reports on in vitro propagation of *Salix tetrasperma* is available. Therefore, the objective of the present investigations was to study the in vitro regeneration potential of *S. tetrasperma* from nodal explants and mass multiplication by manipulation of culture media and Plant Growth Regulators (PGRs).

Materials and methods

Establishment of aseptic culture

Young, green and actively growing shoots of *S. tetrasperma* were collected from a 30-year-old mature plant maintained at the University Department of Botany, Aligarh during the month of August. These were washed under running tap water for 20 min, treated with a laboratory detergent (Labolene, Qualigens, India) 5% (v/v) for 5 min followed by 3–4 washing with sterile distilled water (DW). The plant material was surface sterilized with 0.1% (w/v) $HgCl_2$ for 4 min following repeated washes with sterile distilled water. 0.5–1 cm sized nodal segments were excised aseptically and cultured on sterile shoot induction medium.

Media and culture conditions

The nutrient medium used in all the experiments consisted of Woody Plant Medium (Lloyd and McCown 1981) with 3% (w/v) sucrose (Qualigens, India) supplemented with various concentrations of different PGRs. All the salts used were of analytical grade. The medium was solidified with 0.8% (w/v) bacteriological grade agar (Qualigens, India) and the pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. All the culture vials were incubated in culture room at 25 ± 2°C under 16/8 h (light/dark) cycle with a light intensity of 50 $\mu mol^{-2}\ s^{-1}$ supplied by cool white fluorescent lamps (2 tubes × 40 W, Philips, India) and with 60–65% relative humidity.

Shoot induction and multiplication

Nodal segments were placed on WPM augmented with various cytokinins (BA, Kn and 2 iP) at different concentrations (0.0, 1.0, 2.5, 5.0, 7.5, 10.0 μM) either singly or in combination with auxins (NAA, IAA, IBA) as listed in Tables 1, 2 and 3. Subculturing onto the same medium was performed every 3 weeks. The frequency of explants producing shoots, number of shoots per explants and shoot length were scored after 6 and 12 weeks of culture. Each treatment consisted of 20 replicates and all the experiments were repeated thrice.

In vitro rooting

Healthy and well-elongated shoots (4–5 cm) were excised from culture and transferred to rooting media composed of WPM or 1/2 WPM supplemented with different concentrations (0.0, 0.1, 0.5, 1.0 μM) of either of the auxins (NAA, IAA, IBA) as illustrated in Table 4. Data on percentage of rooting, mean number of roots and root length per shoot were recorded after 4 weeks of transfer to rooting media. Each treatment consisted of 20 replicates and all the experiments were repeated thrice.

Acclimatization

Plantlets with well developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic cups containing sterile Soilrite, garden soil, garden soil-vermiculite (1:1) mixture or vermiculite under diffused light (16:8 h photoperiod) conditions. Potted plantlets were covered with transparent polythene bags to ensure high humidity,

Table 1 Effect of different concentrations of cytokinins on multiple shoot regeneration from nodal segments of *Salix tetrasperma* in WPM, after 6 and 12 weeks of incubation

PGRs (μM)			Response (%)	6 weeks		12 weeks	
BA	Kin	2 iP		Mean no. of shoots	Mean shoot length (cm)	Mean no. of shoots	Mean shoot length (cm)
0.0	0.0	0.0	00	0.0 ± 0.0^{c}	0.0 ± 0.0^{b}	0.00 ± 0.0^{g}	0.00 ± 0.0^{g}
1.0	–	–	80	2.40 ± 0.50^{b}	0.86 ± 0.13^{a}	4.20 ± 1.01^{cde}	1.98 ± 0.40^{c}
2.5	–	–	80	2.60 ± 0.60^{b}	0.88 ± 0.08^{a}	5.60 ± 1.02^{c}	1.20 ± 0.15^{def}
5.0	–	–	90	4.40 ± 0.50^{a}	0.92 ± 0.04^{a}	10.80 ± 0.86^{a}	1.18 ± 0.05^{def}
7.5	–	–	85	2.40 ± 0.60^{b}	0.72 ± 0.11^{a}	5.80 ± 0.58^{c}	0.96 ± 0.15^{ef}
10.0	–	–	85	1.60 ± 0.24^{b}	0.70 ± 0.15^{a}	4.80 ± 1.06^{cd}	0.88 ± 0.15^{f}
–	1.0	–	70	1.80 ± 0.20^{b}	1.10 ± 0.06^{ab}	2.60 ± 0.24^{def}	1.42 ± 0.17^{def}
–	2.5	–	75	2.20 ± 0.37^{ab}	1.14 ± 0.13^{ab}	4.40 ± 0.67^{cde}	1.28 ± 0.29^{def}
–	5.0	–	78	3.00 ± 0.44^{a}	1.36 ± 0.19^{a}	7.80 ± 0.96^{b}	3.48 ± 0.59^{ab}
–	7.5	–	70	2.00 ± 0.44^{ab}	$1.00 \pm 0.05b^{c}$	4.20 ± 0.48^{cde}	1.90 ± 0.14^{c}
–	10.0	–	70	1.60 ± 0.24^{b}	0.74 ± 0.07^{c}	3.20 ± 0.48^{def}	1.80 ± 0.12^{de}
–	–	1.0	60	1.20 ± 0.20^{a}	1.36 ± 0.26^{b}	1.80 ± 0.20^{fg}	1.96 ± 0.23^{c}
–	–	2.5	65	1.40 ± 0.24^{a}	1.62 ± 0.34^{b}	2.20 ± 0.37^{ef}	2.84 ± 0.43^{b}
–	–	5.0	70	2.40 ± 0.74^{a}	2.58 ± 0.16^{a}	4.40 ± 0.60^{cde}	3.78 ± 0.30^{a}
–	–	7.5	70	2.00 ± 0.44^{a}	1.50 ± 0.20^{b}	3.00 ± 0.44^{def}	2.04 ± 0.17^{c}
–	–	10.0	65	1.20 ± 0.20^{a}	1.10 ± 0.23^{b}	2.80 ± 0.37^{def}	1.68 ± 0.24^{def}

Values represent means ± standard error of 5 randomly selected readings of 20 replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

maintained in a growth room and watered with 1/2 WPM solution every alternate day for 2 weeks. Thereafter, the bags were removed in order to harden the plants to field conditions. After 4 weeks, the acclimatized plants were transferred to pots containing normal garden soil and maintained in a greenhouse.

Histological analysis

For histological examination, nodal explants bearing regenerated shoot buds were excised at the time of second subculture and fixed in 5:5:90 (v/v/v) formalin:acetic acid:ethanol (FAA) for 24 h followed by storing in 70% (v/v) ethanol. Tissues were hydrated with a graded ethanol-xylol series followed by paraffin embedding using the method described by Johansen (1940). Longitudinal sections, 10 μm thick, were cut using a Spencer 820 rotatory microtome (American Optical Corporation, Buffalo, NY, USA) and the resulting paraffin ribbons were passed through a series of deparaffinising solutions, followed by staining with safranin and fast green (Johansen 1940). Sections were examined under a light microscope (Olympus CH20i; India Pvt., Ltd., New Delhi).

Statistical analysis

The experiments were based on a completely Randomized Block Design (RBD) with 20 replicates per treatment. The data on various parameters were subjected to one-way Analysis of Variance (ANOVA) using SPSS version 16 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range test at $P = 0.05$. The results were expressed as the means ± SE of three repeated experiments.

Results and discussion

Effect of cytokinins

In the present study, nodal explants cultured on WPM basal without any growth regulator failed to produce shoots, even after 6 weeks of culture necessitating the requirement of cytokinins for multiple shoot induction. Generally, a cytokinin is required for in vitro axillary shoot induction and proliferation. However, its effective type and optimal concentration varies with the system (Park et al. 2008). Cytokinins were reported to play a key role in DNA synthesis and cell division, which might be the reason for induction of multiple shoots. A varied response was observed with different concentrations of different cytokinins (BA, Kn and 2 iP) as summarized in Table 1. WPM supplemented with different concentrations of BA (1.0–10.0 μM) stimulated axillary shoot sprouting after 1 week of culture, and 5.0 μM BA was found to be the optimum for maximum (4.40 ± 0.50 shoots) shoot

Table 2 Combined effect of auxins with the optimal concentration of cytokinins on shoot regeneration from nodal explants of *Salix tetrasperma* after 12 weeks of culture

PGRs (μM)						Response (%)	Mean no. of shoots	Mean shoot length (cm)
BA	Kin	2 iP	NAA	IAA	IBA			
5.0	–	–	0.1	–	–	90	10.40 ± 0.60^{bcdef}	1.18 ± 0.05^{g}
5.0	–	–	0.5	–	–	92	11.40 ± 0.51^{abc}	1.30 ± 0.11^{g}
5.0	–	–	1.0	–	–	90	9.60 ± 0.40^{defgh}	0.98 ± 0.04^{g}
5.0	–	–	–	0.1	–	85	10.20 ± 0.37^{cdefg}	1.02 ± 0.06^{g}
5.0	–	–	–	0.5	–	90	11.20 ± 0.37^{abcd}	1.12 ± 0.03^{g}
5.0	–	–	–	1.0	–	83	9.60 ± 0.40^{defgh}	0.92 ± 0.03^{g}
5.0	–	–	–	–	0.1	78	9.80 ± 0.58^{defgh}	0.92 ± 0.04^{g}
5.0	–	–	–	–	0.5	80	8.80 ± 0.37^{fghi}	1.02 ± 0.02^{g}
5.0	–	–	–	–	1.0	75	9.40 ± 0.68^{efghi}	0.90 ± 0.08^{g}
–	5.0	–	0.1	–	–	80	9.20 ± 0.66^{efghi}	4.10 ± 0.17^{cdef}
–	5.0	–	0.5	–	–	83	12.60 ± 0.51^{a}	4.94 ± 0.05^{a}
–	5.0	–	1.0	–	–	82	9.00 ± 0.83^{cfghi}	4.08 ± 0.38^{cdef}
–	5.0	–	–	0.1	–	75	9.00 ± 0.31^{fghi}	4.02 ± 0.02^{def}
–	5.0	–	–	0.5	–	78	11.80 ± 0.66^{ab}	4.34 ± 0.18^{bcd}
–	5.0	–	–	1.0	–	73	8.80 ± 0.20^{fghi}	3.94 ± 0.06^{def}
–	5.0	–	–	–	0.1	72	8.40 ± 0.81^{hij}	3.92 ± 0.05^{def}
–	5.0	–	–	–	0.5	75	11.20 ± 0.58^{abcd}	4.30 ± 0.14^{bcde}
–	5.0	–	–	–	1.0	70	8.40 ± 0.40^{hij}	3.80 ± 0.09^{f}
–	–	5.0	0.1	–	–	70	7.00 ± 0.54^{jkl}	4.30 ± 0.18^{bcde}
–	–	5.0	0.5	–	–	72	8.80 ± 0.58^{fghi}	4.68 ± 0.22^{ab}
–	–	5.0	1.0	–	–	70	6.00 ± 0.31^{lm}	4.06 ± 0.09^{def}
–	–	5.0	–	0.1	–	67	6.80 ± 0.20^{klm}	3.98 ± 0.03^{def}
–	–	5.0	–	0.5	–	70	8.60 ± 0.24^{ghi}	4.48 ± 0.04^{bc}
–	–	5.0	–	1.0	–	65	6.00 ± 0.31^{lm}	3.88 ± 0.05^{ef}
–	–	5.0	–	–	0.1	63	6.60 ± 0.24^{klm}	3.82 ± 0.15^{f}
–	–	5.0	–	–	0.5	67	7.80 ± 0.37^{ijk}	4.28 ± 0.11^{bcde}
–	–	5.0	–	–	1.0	62	5.40 ± 0.40^{m}	3.76 ± 0.13^{f}

Values represent means ± standard error of 5 randomly selected readings of 20 replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

induction after 6 weeks of incubation (Fig. 1a). Further increase in BA concentration beyond 5.0 μM, did not show any improvement in shoots induction, rather a decreasing trend in all the parameters evaluated was evident. The reduction in the regeneration potential appeared to be due to the detrimental effect of high concentration on the cells predetermined to form vegetative buds. However, the shoots regenerated on BA supplemented WPM were stunted and no elongation was observed even after 12 weeks of culture. Upon exposure at higher concentrations of BA (5.0 μM and above) beyond 6 weeks and up to a maximum exposure duration of 12 weeks, the regenerated shoots tend to show hyperhydricity. Hyperhydricity of shoots exposed to higher concentrations of BA has been reported earlier by Amo Marco and Lledo (1996) on *Salix tarraconensis* and by Read et al. (1989) for some clones of *Salix viminalis*. When Kn was used as the sole source of cytokinin, a slight basal callusing was observed with a low regeneration frequency (Table 1). A maximum of 3.00 ± 0.44 shoots with the highest percentage (78%) of responding explants was produced at 5.0 μM Kn after 6 weeks of incubation. These results clearly showed that Kn was inferior to BA in this species which is in accordance with earlier reports on many woody plant species (Anis et al. 2010; Ahmad et al. 2008). However, in this case, the shoots exhibited a better elongation as compared to those induced using BA; 2 iP was also used for shoot induction and comparatively few shoots were formed with a low regeneration frequency, the highest being 2.40 ± 0.74 shoots with 70% response at 5.0 μM concentration after 6 weeks of culture, but the elongation of regenerated shoots was maximum compared to BA or Kn (Table 1), indicating a tendency of the regenerated shoots towards apical dominance. Among the three cytokinins

Table 3 Multiplication and elongation of BA (5.0 μM) treated shoot clusters on WPM using lower concentrations of BA and auxins after 12 weeks of culture

BA (μM)	NAA (μM)	IAA (μM)	IBA (μM)	Response (%)	Mean no. of shoots	Mean shoot length (cm)
0.0	0.0	0.0	0.0	80	10.40 ± 0.81^{de}	3.06 ± 0.64^{e}
0.5	–	–	–	80	13.60 ± 0.87^{bc}	3.76 ± 0.39^{cde}
1.0	–	–	–	90	14.40 ± 1.07^{b}	4.76 ± 0.35^{abc}
2.0	–	–	–	75	11.20 ± 0.37^{de}	3.90 ± 0.10^{bcde}
1.0	0.1	–	–	92	15.20 ± 0.37^{b}	4.80 ± 0.37^{ab}
1.0	0.5	–	–	95	18.40 ± 0.92^{a}	5.30 ± 0.43^{a}
1.0	1.0	–	–	90	15.00 ± 0.54^{b}	4.64 ± 0.26^{abcd}
1.0	–	0.1	–	85	10.80 ± 0.37^{de}	4.72 ± 0.19^{abcd}
1.0	–	0.5	–	91	12.00 ± 0.70^{cd}	4.78 ± 0.09^{ab}
1.0	–	1.0	–	83	9.80 ± 0.37^{e}	4.32 ± 0.12^{abcd}
1.0	–	–	0.1	81	10.80 ± 0.58^{de}	4.00 ± 0.08^{bcde}
1.0	–	–	0.5	85	11.20 ± 0.58^{de}	4.10 ± 0.06^{bcd}
1.0	–	–	1.0	80	10.20 ± 0.37^{de}	3.74 ± 0.16^{de}

Values represent means ± standard error of 5 randomly selected readings of 20 replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

Table 4 In vitro rooting of regenerated shoots of *Salix tetrasperma* using different treatments after 4 weeks of transfer

Media	PGRs (μM)			Response (%)	Mean no. of roots	Mean root length (cm)
	IBA	IAA	NAA			
WPM	–	–	–	50	1.20 ± 0.20^{e}	0.52 ± 0.02^{g}
1/2 WPM	–	–	–	65	2.20 ± 0.37^{de}	0.82 ± 0.04^{de}
1/2 WPM	0.1	–	–	85	4.20 ± 0.48^{c}	0.98 ± 0.04^{bc}
1/2 WPM	0.5	–	–	95	8.20 ± 0.66^{a}	2.16 ± 0.06^{a}
1/2 WPM	1.0	–	–	83	6.20 ± 0.66^{b}	1.08 ± 0.03^{b}
1/2 WPM	–	0.1	–	70	3.20 ± 0.20^{cd}	0.98 ± 0.04^{bc}
1/2 WPM	–	0.5	–	72	5.40 ± 0.40^{b}	1.14 ± 0.06^{b}
1/2 WPM	–	1.0	–	70	2.60 ± 0.24^{d}	0.88 ± 0.04^{cd}
1/2 WPM	–	–	0.1	75	2.20 ± 0.20^{de}	0.68 ± 0.04^{ef}
1/2 WPM	–	–	0.5	80	3.00 ± 0.00^{cd}	1.02 ± 0.08^{bc}
1/2 WPM	–	–	1.0	75	3.00 ± 0.31^{cd}	0.54 ± 0.20^{fg}

Values represent means ± standard error of 5 randomly selected readings of 20 replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

tried, BA was the most effective followed by Kn and 2 iP which is in accordance with earlier report on hybrid willow (*S. matsudana* × *alba* NZ-1002) by Bhojwani (1980). The effectiveness of BA on multiple shoot bud differentiation has also been demonstrated in a number of cases using a variety of explants (Anis et al. 2009; Husain and Anis 2009; Siddique and Anis 2009).

The differentiation of shoot buds was confirmed by histological examination of cultured explants. Most shoot buds revealed no visible connection with the original vascular tissue, although they appeared to have originated from the meristematic zone beneath the epidermis. It is possible that the initial shoots developed from pre-existing meristems, as seen in histological slides (Fig. 1c).

Multiplication and proliferation of shoots

In vitro raised shoots of *S. tetrasperma* multiplied and elongated onto the same medium when either Kn or 2 iP was used as supplement to WPM. The optimum proliferation response was recorded at 5.0 μM for both the PGRs yielding 7.80 ± 0.96 and 4.40 ± 0.60 shoots, respectively, after 12 weeks of culture (Table 1). BA at lower concentrations (0.5, 1.0 and 2.0 μM) were effective for multiplication and proliferation. However, at higher concentrations (2.5 or above), the shoots failed to elongate even though the number of shoots continued to increase thereby forming shoot clusters. For this, a different strategy was adopted and the induced shoots were transferred to WPM

Fig. 1 **a** Multiple shoots induction on WPM + BA (5.0 μM) after 6 weeks of culture. Inset—an excised nodal segment **b** Shoot multiplication on WPM + BA (1.0 μM) after 12 weeks of culture. **c** LS of shoot buds showing leaf primordial and vascular bundles (*LP* leaf primordia, *VB* vascular bundles, *SA* shoot apex; Bar = 200 μm) **d** Mass multiplication WPM + BA (1.0 μM) + NAA (0.5 μM) after 12 weeks of culture. **e** Rooting of in vitro raised shoots on 1/2 WPM + IBA (0.5 μM)

containing lower concentrations of BA (0.5, 1.0 and 2.0 μM) after 6 weeks exposure. At the end of 12 weeks, healthy and well-elongated shoots were formed, the maximum being at 1.0 μM BA. The decrease in the concentration of BA from 5.0 μM in explants establishment stage to 1.0 μM BA in multiplication stage improved shoot proliferation and nodal segments which were earlier exposed to 5.0 μM BA for 6 weeks yielded 14.40 ± 1.07 shoots with a mean shoot length of 4.76 ± 0.35 cm after 12 weeks on transfer to a multiplication medium containing 1.0 μM BA (Table 3). This increase in shoot proliferation on lowering the concentration of BA may be due to the residual cytokinin present in the shoots at multiplication stage (Shukla et al. 2009). Such a stimulating effect of lower concentrations of BA on shoot elongation and proliferation was well documented in earlier reports on some *Salix* species by Bergman et al. (1985) and Read et al. (1989). Similarly Lyyra et al. (2006) also achieved shoot elongation in *Salix nigra* upon transfer of shoot buds to one tenth of the concentrations of PGRs used in the callus initiation medium. These results clearly suggested that a continuous high dosage of either Kn or 2 iP was required for shoot multiplication in the species investigated while for BA, lowering the concentration proved beneficial for further growth and proliferation.

Effect of combined treatments of cytokinins along with an auxin was evaluated for shoot multiplication rate by taking the optimized concentration of different cytokinins in combination with different concentrations (0.1, 0.5, 1.0 μM) of auxins (NAA, IAA, IBA). For shoot proliferation, the reduced concentration of BA (1.0 μM) along with NAA (0.5 μM) was more effective over others (Tables 2, 3). Shoot clusters regenerated on WPM containing 5.0 μM BA proliferated and produced 18.40 ± 0.92 well-elongated shoots upon transfer to a medium containing BA (1.0 μM) and NAA (0.5 μM) (Table 3; Fig. 1b). Such stimulating effects of combinations of these two hormones are well supported by earlier studies on *Balanites aegyptiaca* (Anis et al. 2010) and *Vitex negundo* (Ahmad et al. 2008; Ahmad and Anis 2010). Combinations of auxins along with Kn (5.0 μM) or 2 iP (5.0 μM) also yielded favorable results and multiplication

and elongation of shoots were enhanced with an optimum response at 0.5 μM of NAA supplementation (Table 2). However, combination of 5.0 μM BA with auxins (NAA, IAA and IBA) at different levels (0.1, 0.5, 1.0 μM) did not significantly improve the regeneration and number of shoots and shoot length were not significantly affected (Table 2). Effectiveness of auxins for stimulating axillary shoot proliferation synergistically with cytokinins (BA, Kn, and 2 iP) follows the order NAA > IAA > IBA (Tables 2, 3).

Rooting

Rooting of in vitro raised shoots were carried out by transferring the healthy, well-elongated shoots to rooting media composed of either full-strength or half-strength WPM with or without different concentrations of auxins (NAA, IAA, IBA). Half-strength growth-regulator-free WPM was found superior to full-strength WPM for root development as this was able to induce 2.0 roots per shoot as against 1.0 in case of full-strength (Table 4). Rooting of willow species in hormone-free WPM was earlier reported by Gebhardt (1992) and Park et al. (2008). The production of adventitious roots on auxin free basal media may be due to the endogenous level of salicylic acid which plays an important role in plant growth and development (Raskin 1992) including in vitro rooting (Khalafalla and Hattori 2000). Of the various treatments evaluated for in vitro rooting, the best rooting response could be observed in half-strength WPM containing 0.5 μM IBA where about eight roots were formed with a length of about 5 cm (Table 4; Fig. 1e). Similar results were reported in *Tylophora indica* by Faisal et al. (2007).

Hardening and acclimatization

The rooted plantlets were successfully hardened off inside the culture room in selected planting substrate for 4 weeks and eventually established in garden soil in the green house. Among the tested potting substrates, Soilrite™ (Keltech Energies Ltd, Bangalore) was found to be the most suitable for the successful establishment (Fig. 2) of *S. tetrasperma* plantlets where maximum (85%) survival rate was recorded (Table 5). The plantlets were subsequently transferred to pots containing garden soil followed by shifting to green house and finally to field conditions under full sun.

Conclusion

The present study demonstrates the successful use of nodal explants from an elite clone of *S. tetrasperma* for multiple shoot induction and proliferation. Nodal segments carry a high potential for rapid shoot regeneration on a medium containing 5.0 μM BA followed by transfer to a medium containing lower concentrations of PGRs viz., 1.0 μM BA and 0.5 μM NAA after 6 weeks, thereby reducing the input cost. Such a system would be useful for mass propagation and multiplication of this valuable plant species. Since the regeneration of plantlets was achieved without an intervening callus phase, somaclonal variation if any may be avoided and thus ensures clonal multiplication. Commercial exploitation of the developed protocol is possible, as the nodal segments from in vitro raised shoots may be employed as propagules for further multiplication, obviating the dependence on field material.

Fig. 2 An acclimatized plant in Soilrite (4 weeks old)

Table 5 Effect of different potting substrates on the survival of plantlets of *Salix tetrasperma* after 4 weeks of transfer

Potting substrate	Survival (%)
Soilrite	85.00 ± 1.61[a]
Vermiculite	69.80 ± 0.80[c]
Garden soil	56.60 ± 0.92[d]
Vermiculite-garden soil mixture (1:1)	74.60 ± 1.56[b]

Values represent means ± standard error of five randomly selected readings of 20 replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test

Acknowledgments Financial assistance to Md Imran Khan in the form of JRF (NET) by Council of Scientific and Industrial Research (CSIR), Govt. of India, New Delhi is gratefully acknowledged. Research support provided by the Department of Science and Technology (DST) and University Grants Commission (UGC) Govt. of India, New Delhi, in the form of DST-FIST (2005) and UGC-SAP (2009) Programmes is duly acknowledged.

References

Agrawal DC, Gebhardt K (1994) Rapid micropropagation of hybrid willow (*Salix*) established by ovary culture. J Plant Physiol 143:763–765

Ahmad N, Anis M (2007) Rapid clonal multiplication of a woody tree, *Vitex negund*o L. through axillary shoots proliferation. Agroforest Syst 71:195–200

Ahmad N, Anis M (2010) An efficient in vitro process for recurrent production of cloned plants of *Vitex negundo* L. Eur J For Res. doi: 10.1007/s10342-010-0415-y

Ahmad N, Wali SA, Anis M (2008) In vitro production of true-to-type plants of *Vitex negundo* L. from nodal explants. J Hortic Sci Biotech 83(3):313–317

Ahuja MR (1993) Regeneration and germplasm in aspen—*Populus*. In: Ahuja MR (ed) Micropropagation of woody plants. Kluwer, Dordrecht, pp 187–194

Amo Marco JB, Lledo MD (1996) In vitro propagation of *Salix tarraconensis* Pau ex Font Quer, an endemic and threatened plant. In Vitro Cell Dev Biol Plant 32:42–46

Anis M, Husain MK, Faisal M, Shahzad A, Ahmad N, Siddique I, Khan H (2009) In vitro approaches for plant regeneration and conservation of some medicinal plants. In: Kumar A, Sopory SK (eds) Recent advances in plant biotechnology and its applications, vol 14. IK International Pvt Ltd, New Delhi, pp 397–410

Anis M, Varshney A, Siddique I (2010) In vitro clonal propagation of *Balanites aegyptiaca* (L.) Del. Agroforest Syst 78:151–158

Anonymous (2003) The wealth of India: a dictionary of Indian raw materials and industrial products, CSIR, New Delhi, 169–175

Bergman L, von Arnold S, Erickson T (1985) Effects of N^6-benzyladenine on shoots of five willow clones (*Salix* spp) cultured in vitro. Plant Cell Tissue Organ Cult 4:135–144

Bhojwani SS (1980) Micropropagation method for a hybrid willow (*Salix matsudana* X *alba* NZ-1002). N Z J Bot 18:209–214

Chalupa V (1983) Micropropagation of conifer and broad leaved forest trees. Commun Instit Forest Cechoslov 13:7–39

Dhir KK, Angrish R, Bajaj M (1984) Micropropagation of *Salix babylonica* through in vitro shoot proliferation. Proc Indian Acad Sci (Plant Sci) 93(6):655–660

Faisal M, Anis M (2002) Rapid in vitro propagation of *Rauvolfia tetraphylla* L.—an endangered medicinal plant. Physiol Mol Biol Plants 8:295–299

Faisal M, Ahmad N, Anis M (2007) An efficient micropropagation system for *Tylophora indica*: an endangered, medicinally important plant. Plant Biotechnol Rep 1:765–772

Gebhardt K (1992) Grudlagen der Zuhtung pharmazeutisch wertvoller Weiden. Die Holzzucht 46:9–14

Giri CC, Shyamkumar B, Anjaneyulu C (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. Trees 18:115–135

Granel T, Robinson B, Mills T, clothier B, Green S, Fung L (2002) Cadmium accumulation by willow clones used for soil conservation, stock fodder, and phytoremediation. Aust J Soil Res 40:1331–1337

Grönroos L, von Arnold S, Erikson T (1989) Callus production and somatic embryogenesis from floral explants of basket willow (*Salix viminalis* L.). J Plant Physiol 134:558–566

Husain MK, Anis M (2009) Rapid in vitro multiplication of *Melia azaderach* L. (A multipurpose woody tree). Acta Physiol Plant 31:765–772

Johansen DA (1940) Plant microtechnique. McGraw-Hill, New York, pp 126–154

Khalafalla MM, Hattori K (2000) Ethylene inhibitors enhance in vitro root formation on faba bean shoots regenerated on medium containing thidiazuron. Plant Growth Regul 32:59–63

Kuzovkina YA, Quigley MF (2005) Willows beyond wetlands: uses of *Salix* species for environmental projects. Water Air Soil Pollut 162:183–204

Lloyd G, Mc Cown B (1981) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Proc Int Plant Propag Soc 30:421–427

Lyyra S, Lima A, Merkle SA (2006) In vitro regeneration of *Salix nigra* from adventitious shoots. Tree Physiol 26:969–975

Neuner H, Beiderback R (1993) In vitro propagation of *Salix caprea* L by single node explants. Silvae Genet 42:308–310

Park SY, Kim YW, Moon HK, Murthy HN, Choi YH, Cho HM (2008) Micropropagation of *Salix pseudolasiogne* from nodal explants. Plant Cell Tissue Organ Cult 93:341–346

Perttu KL, Kowalik PJ (1997) *Salix* vegetation filters for purification of waters and soils. Biomass Bioenergy 12:9–19

Raskin I (1992) Role of salicylic acid in plants. Annu Rev Plant Physiol Mol Biol 43:439–463

Read PE, Garton S, Tormala T (1989) Willows (*Salix* spp.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry. Trees II, vol 4. Springer, Berlin, pp 370–386

Shukla S, Shukla SK, Mishra SK (2009) In vitro plant regeneration from seedling explants of *Stereospermum personatum* DC: a medicinal tree. Trees 23:409–413

Siddique I, Anis M (2009) Direct plant regeneration from nodal explants of *Balanites aegyptiaca* L. (Del.)—a valuable medicinal tree. New Forests 37:53–62

Stoehr MU, Cai M, Zsuffa L (1989) In vitro plant regeneration via callus culture of mature *Salix exigua*. Can J For Res 19:1634–1638

Thorpe TA, Harry IS, Kumar PP (1991) Application of micropropagation to forestry. In: Debergh PC, Zimmerman RH (eds) Micropropagation. Kluwer, Dordrecht, pp 311–336

Vervaeke P, Luyssaert S, Mertens J, Meers E, Tack FMG, Lust N (2003) Phytoremediation prospects of willow stands on contaminated sediment: a field trial. Environ Pollut 126:275–282

Vinocur B, Carmi T, Altman A, Ziv M (2000) Enhanced bud regeneration in aspen (*Populus tremula* L.) roots cultured in liquid media. Plant Cell Rep 19:1146–1154