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MICROPROPAGATION AND CONSERVATION OF THREE *JUNIPERUS* SPECIES (CUPRESSACEAE)

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Abstract

Shoot tips of three species *Juniperus excelsa* M. Bieb, *Juniperus horizontalis* Moench and *Juniperus chinensis* L. *were* initiated to develop rapidly multiplying protocol for large scale micro propagation, regeneration and conservation of these species. The effect of various concentrations of different plant growth regulators on different media was checked to optimize the efficient media for propagation. The young shoot tips were cultured on MS, WPM and N6 media supplemented with different concentrations of IAA, IBA, 2, 4-D in combination with BAP. The explants used for callus induction in WPM supplemented with 0.50 mg/l 2, 4-D and BAP produced best callus. At this concentration rich solid mass of greenish brown callus was produced in all three species. Large numbers of shoots were also produced in WPM medium containing 0.50mg/l BAP, average shoots produced were *J. excelsa* 6 shoots (6.1±0.2cm), *J. horizontalis* 8 shoots (8.2±0.2cm) while significantly high number was produced by *J. chinensis* with 9 shoots (9.5 ±.0.3cm) in four months time. Rooting also started in all species on the same media when plant attained height of 4-7cm but these roots were not good enough to support plantlet. WPM with IAA and IBA hormones were used for rooting. Best roots were produced in IBA at 1.0mg/l. Significantly high number of roots were produced in *J. chinensis.* After proper production of roots in 6-8 weeks plants were acclimatized and shifted to green house. The survival rate after two months of hardening in green house for *J. excelsa* was 42%, *J. horizontalis* 68% and for *J. chenensis* 87%.

Introduction

Balochistan, (Paksitan) has approximately 86,000 ha of Juniper forests in Ziarat and Loralai Districts. The evergreen *Juniperus excelsa* forest is the second largest forest of the world. This is the world oldest living species on the earth also known as living fossils. The Juniper forest is the only sources of fuel and timber for the local residents of the area due to which extensive areas have been cut over. The forest is threatened due to die back disease, dwarf mistletoe and a large number of other biotic and abiotic factors (Zaidi *et al*., 2008). Its berries and oil are used for medicinal purposes. These forests are also heavily used for grazing of sheep and goats, especially during the summer. The growth rate of tree is very slow, seeds have a very low potential of regeneration, if saved from people and predators. Khattak & Sheikh (1981) worked on dry-zone aforestation in the Juniper forests of Baluchistan. Sheikh (1981) also separated filled and empty Juniper seed by water flotation method. Javed *et al*., (1980) first reported *In vitro* cultivation of *J. excelsa* however they were unable to achieve much success. Negussie (1997) successfully tried *In vitro* induction of multiple buds on excised cotyledons of *J. excelsa*. Conventional breeding of woody trees is a slow and difficult process due to elevated levels of heterozygosity and extended regeneration cycles (Sriskanndarajah *et al*., 1994). Micropropagation is an alternative method of vegetative reproduction. *In vitro* propagation techniques in forestry are useful for mass reproduction of superior trees found in nature, various methods of micropropagation, axillary shoot proliferation has been found to be the most successful for achieving plantlet regeneration from mature forest trees (Von Arnold & Eriksson, 1981). Therefore to overcome the problem of deforestation and for conservation and rehabilitation of Juniper forest Ziarat present study was initiated to develop rapidly multiplying protocol for large scale micro propagation.

Materials and Methods

Young shoot tips of *J. excelsa* were collected from Ziarat, Balochistan while *J. horizontalis and J. chinensis* were collected from Botanic garden University of Balochistan, Quetta these were used as explants. The shoots were washed thoroughly under the running tap water for 10-20 minutes. The stems of explants were soaked in a jar containing distilled water with 0.1% mercuric chloride and 2-3 drops of tween 20 for sterlization for 2 minutes. The explants were washed three times with autoclaved distilled water to remove all the traces of mercuric chloride. The shoot tips were cut into small pieces approximately 2 cm long were used as explants for shoot initiation under *in-vitro* conditions in 250 ml glass jars.

Murashige & Skoog, (MS, 1962), Woody Plant Medium (WPM, Lloyd & McCown, 1980), N6 media supplemented with different concentrations of 2, 4-D in combination with Bezylaminepurine (BAP) 5% sucrose was used as a carbon source. MS, WPM and N6 were supplemented with 5 combinations of auxins and cytokinins for callus induction (Tables 1-4). Young shoots were placed in medium for the induction of callus and shoot/ root formation. WPM medium with different concentrations of Indole Acetic Acid (IAA) and Indole butaryic acid (IBA) were used for rooting (Table 6). The pH of the media was adjusted to 5.7; it was sterilized by autoclaving at 121ºC for 20 min. Cultures were maintained in growth room at 25±2 with light provided by

white inflorescent tube lights for 16 h photoperiod. Glass jars were filled with 25ml media and left in the growth room for two days to check for any contamination after that inoculation of explants was done. 30 explants were used in each treatment and the experiment was repeated five times.

Data was recorded and analyzed for the frequency of the callus formation, callus proliferation, shoot and root size and number. Results are presented as means± Standard Deviation. Explants were first sub cultured weekly to remove phenols than sub culturing was done after 4 weeks, and after 8 weeks on the same hormonal combination and concentration for callus induction.

The plantlets with proper roots were selected for acclimatization Plantlets were removed from culture and roots were gently washed with distilled water to remove the residual gel or medium. These plants were transplanted individually in small pots with soil + peat moss + fertilizer and kept in the lab under controlled light and temperature. After 2-3 weeks of acclimatization and hardening, plants were shifted to conservatory. Data was recorded to determine survival rate after acclimatization.

Effect of growth regulators on callus formation of three *Juniperus species* using different media

Table 1. Response of BAP and 2,4-D using MS media.

Name of media	BAP/2,4-D Conc. (mg/liter)	Average % of callus induction	*J. excelsa*	*J. horizontalis*	*J. chinensis*
MS	0	0	+	++	+
MS	0.25 + 0.25	20	+	++	+
MS	0.50 + 0.50	50	+++	+++	+++
MS	0.75 + 0.75	30	++	++	++
MS	0.1 + 0.1	20	++	++	++

Callus formation: + = Poor: ++ Fair: +++ = Good. 30 explants were used for each treatment; experiment was repeated five times: Results are expressed as mean ± SD. Growth of callus after 8 weeks

Table 2. Response of BAP and 2,4-D using WPM.

Name of media	BAP/2,4-D (mg/liter)	% of callus induction	*J. excelsa*	*J. horizontalis*	*J. chinensis*
WPM	0	30	+	+	+
WPM	0.25+0.25	40	++	++	++
WPM	0.50+0.50	90	++++	++++	+++
WPM	0.75+0.75	70	+++	++	+++
WPM	0.1+ 0.1	60	++	++	++

Callus formation: + = Poor: ++ Fair: +++ = Good. 30 explants were used for each treatment; experiment was repeated five times. Results are expressed as mean ± SD. Growth of callus after 8 weeks

Table 3. Response of BAP and 2,4-D using N6 media.

Name of media	BAP/2,4-D Conc. (mg/liter)	% of callus induction	*J. excelsa*	*J. horizontalis*	*J. chinensis*
N6	0	0	-	-	+
N6	0.25	30	++	+	+
N6	0.50	60	++	+++	+++
N6	0.75	40	++	++	++
N6	0.1	20	+	++	++

- No callus formation, + = Poor: ++ Fair: +++ = Good

30 explants were used for each treatment; experiment was repeated five times. Results are expressed as mean ± SD.

Growth of shoots after 8 weeks

Table 4. Shoot regeneration from callus in *Juniperus species* using BAP and WPM.

Name of media	BAP Conc. (mg/liter)	% of shoot induction and proliferation		*J. chinensis*	Callus induction and proliferation		
		J. excelsa	*J. horizontalis*		*J. excelsa*	*J. horizontalis*	*J. chinensis*
WPM	0	22	35	38	++	++	++
WPM	0.25	54	66	65	+++	+++	+++
WPM	0.50	90	100	100	++++	++++	++++
WPM	0.75	86	90	92	+++	+++	+++
WPM	0.1	30	33	45	+	++	++

Growth after 3 months. + = Poor: ++ Fair: +++ = Good

30 explants were used for each treatment; experiment was repeated five times. Results are expressed as mean ± SD

Table 5. Effect of different concentrations of BAP using WPM for shoot and root regeneration.

Name of Media	BAP (mg/liter)	Number of shoots			Number of roots		
		J. excelsa	*J. horizontalis*	*J. chinensis*	*J. excelsa*	*J. horizontalis*	*J. chinensis*
WPM	0	4.5 ± 2	5.4 ± 4	5.8 ± 1	1.2 ± 3	2.25 ± 1	3.0 ± 2
WPM	0.25	5.2 ± 2	6.8 ± 3	8.0 ± 2	1.7 ± 1	2.75 ± 1	3.0 ± 1
WPM	0.50	6.1 ± 2	8.2 ± 2	9.5 ± 2	3.5 ± 1	4.8 ± 2	5.5 ± 2
WPM	0.75	6.0 ± 2	7.9 ± 1	9.2 ± 2	2.0 ± 2	3.75 ± 1	4.15 ± 1
WPM	0.1	4.2 ± 1	7.1 ± 3	8.5 ± 1	1.0 ± 5	3.25 ± 2	4.18 ± 2

30 explants were used for each treatment; experiment was repeated five times. Results are expressed as mean ± SD. Growth of shoots after 4 months

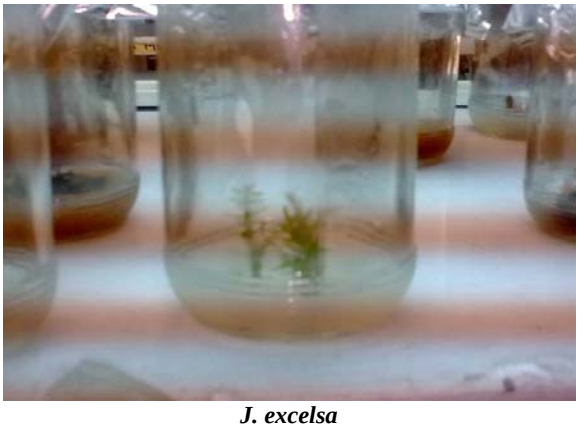
Table 6. Effect of different concentrations of IAA and NAA on rooting of *Juniperus* species in WPM.

Hormone + WPM medium	Conc. (mg/liter)	Number of roots		
		J. excelsa	*J. horizontalis*	*J. chinensis*
IBA	0	1.8 ± 0.02	2.2 ± 0.03	1.6 ± 0.01
	0.25	2.0 ± 0.3	3.0 ± 0.5	2.4 ± 0.2
	0.50	3.6 ± 0.2	5.20 ± 0.4	3.5 ± 0.2
	0.75	4.1 ± 0.2	7.5 ± 0.2	5.1 ± 0.2
	0.1	6.4 ± 0.3	7.9 ± 0.2	9.5 ± 0.5
	1.50	6.0 ± 0.2	6.4 ± 0.1	7.8 ± 0.3
IAA	0	0.6 ± 0.02	1.2 ± 0.02	1.5 ± 0.03
	0.25	1.0 ± 0.2	2.2 ± 0.3	1.8 ± 0.4
	0.50	3.20 ± 0.5	4.2 ± 0.2	2.6 ± 0.3
	0.75	3.15 ± 0.2	4.7 ± 0.2	4.0 ± 0.3
	0.1	2.65 ± 0.2	4.6 ± 0.3	3.6 ± 0.2
	1.50	2.00 ± 0.3	3.9 ± 0.1	2.8 ± 0.2

Roots were produced after 4-6 weeks in 4 months old tissue cultured shoots: 10 shoots were used for each treatment: Experiment was repeated five times. Results are expressed as mean ± SD

J. horizontalis

J. chinensis



J. excelsa

Fig. 1. Effect of 0.50mg/l of BAP using WPM for shoot formation.

Results and Discussion

To optimize the medium for regeneration and proliferation of callus, shoots and roots formation different concentration of growth hormones were used. The young shoot tips were used as explants for callus induction. Data presented in Tables 1-3 shows effect of four different combinations of phyto-homones supplemented in MS, WPM and N6 media. Explants were able to successfully produce greenish-brown callus in 6-8 weeks. Significantly better callus was obtained in WPM medium with 0.50 mg/l Concentration of BAP and 0.50 mg/l, 2, 4-D for callus induction (Table 3). Increase or decrease in the concentration of hormone adversely affected the rate of callus formation and growth. Comparatively low callus production was observed in MS and N6 medium supplemented with same combination of hormones. Contrary to our results Negussie (1997) reported that embryo explants showed the best response when the same concentration of NAA was added to Murashige and Skoog medium containing BAP.

The rate of regeneration was achieved with simple WPM but it was accelerated when WPM was supplemented with four different concentration and combination of BAP. The concentration of BAP at 0.50mg/l in WPM proved to be the best for early initiation, callus formation and shoot proliferation (Table 4). Maximum growth of callus was observed in eight to twelve weeks. The proliferation response increased with sub culturing and the highest proliferation response was obtained in 4th subculture. *J. chinensis* and *J. horizontalis* response was rapid than *J. excelsa*.

Maximum increase in shoot number and length was also observed with WPM medium at BAP 0.50mg/l (Table 5). After enough elongation of shoots in 12-16 weeks rooting also started in the same medium when the shoots attained the height of almost 4cm. Significantly high number of shoots (7-9 and roots 3-5) was found in *J. chinensis* than *J. horizontalis*, (6-8 shoots and 2-4 roots were produced, minimum number of shoots (4-6 and roots 1-3) was observed in *J. exclesa* (Table 5). Therefore WPM with 0.5mg/l BAP proved to be very good for shoot and root formation in *in-vitro* cultivation of *Juniperus* species, its growth was inhibited by increasing the concentration of BAP to 0.75-1mg/l (Table 5). Similar results were found by Campbell and Dunan (1975) while working on *Picea* species.

Although roots also appeared on the same medium but these were few, weak and not enough long to support plantlet which became a major problem for rooting. Therefore WPM was supplemented with five concentrations of IAA and IBA to check their effect on rooting. Maximum numbers of roots were achieved after 4-6 weeks (9.5±0.5/ plantlet) in *J. chinensis*, than *J. horizontalis* produced (7.9±0.2 roots/plantlet) and while lowest number of roots was observed in *J. excelsa* (6.4±0.3 roots/plantlet). Significantly high numbers of roots were produced at 1 mg/l IBA (Table 6), Negussie (1997) also found that further increase in hormone concentration inhibited root growth. Biondi *et al*., (1984) also observed that higher concentrations of IAA promoted shoot-tip necrosis.

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