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Nitrogen Effect on Callus Induction and Plant Regeneration of *Juniperus excelsa*

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ABSTRACT

Nitrogen plays a major role in growth and differentiation such as stem elongation and leaf morphology. Both form and amount of nitrogen in *in vitro* medium have significant effects on rate of cell growth and differentiation. Calli and adventitious buds were initiated from excised shoot cuttings obtained from 8-year-old young plants. Murashige and Skoog medium (MS) was used as basal medium containing full and half strength MS nitrogen, only nitrate (KNO_3) or ammonium nitrate (NH_4NO_3) with or without supplementation by glutamine (100 mg L^{-1}). Full strength MS inhibited active callus production that can be minimized by lowering amount of ammonium or total nitrogen. Medium with half strength MS nitrogen stimulated better callus production that can be increased by glutamine supplementation. Nitrogen supplimentation only as nitrate produced no callus, but adventitious bud were formed from callus as well as from leaf lamina, directly, suggesting that nitrate can release lateral buds from apical dominance. Therefore, in tissue culture if the aim is plant propagation, shoots could get in shorter time by elimination of ammonium salt from medium.

Key Words: *Juniperus excelsa;* Nitrogen source; Tissue culture; Plant growth regulators

INTRODUCTION

Junipers are evergreen shrubs or trees belonging to the family *Cupressacea.* Duhoux and Norreel (1973) first time reported *in vitro* propagation of *Juniperus excelsa.* Thereafter, several other researches have published their work (Javeed *et al.*, 1980; Mahmood *et al.*, 1992; Sadri & Naraghi, 1995; Negussie, 1997), however, only Negussie (1997) successfully induced shoots in plants.

Nitrogen assimilation and its role in plant growth and development are important in establishing understanding of cell differentiation in plants. Both the form and amount of nitrogen in the *in vitro* medium have significant effects on rate of cell growth, differentiation and cell totipotency (Kirby *et al.*, 1987).

In tissue culture medium nitrate, ammonium salt, amino acids and complex organic products supply nitrogen. Nitrate is good source of nitrogen because it is readily taken up and metabolized by the cells and affects a number of developmental processes leading to root branching, breaking of seed and bud dormancy and release of apical dominance. A reduction in nitrogen application often initiates sexual development (Trewavas, 1983).

Medium with nitrate as the only source of nitrogen often becomes more alkaline in time, a tendency that can be controlled by adding a small amount of ammonium salt. Control of pH is not the only reason for using both nitrate and ammonium in the medium but ammonium/nitrate balance stimulates morphogenesis and embryogenesis. However, one has to be careful with ammonium being toxic (Bonga & Von Aderkas, 1992).

In this research the effects of nitrogen source (inorganic and organic types) were studied on callus induction and *in vitro* shoot proliferation of *J. excelsa.*

MATERIALS AND METHODS

Callus cultures were initiated from shoot cuttings obtained from 8-year-old plants. The shoots were surface sterilized as shoots were put for (i) 30 min in water; (ii) 30 min in washing liquid solution (Tween 80 solution 1%), (iii) 30 min in water; (iv) 60 min in fungicide, Benomil 2%, followed by washing with sterile water, (v) 30 sec in 70% alcohol followed by washing with sterile water, (vi) 10 min in 0.1% $HgCl_2$ followed by washing with sterile water 4 times, (vii) 10 min in 60% Sodium hypo chlorite (NaOCl) detergent, followed by washing with sterile water four times.

Shoots with apical or lateral buds and needles were cultured on MS medium (Murashige & Skoog, 1962) and 6–modified MS media (X, Y, Z, W, V, U), which differ in nitrogen sources i.e., NH_4NO_3, KNO_3 and Glutamine (Table

Table I. Nitrogen source of MS and 6 MS modified media (cited amounts are in mgL^{-1})

N-source / Media	MS	X	Y	Z	W	V	U
NH_4NO_3	1650	0	0	1650	1650	825	825
KNO_3	1900	1900	1900	0	0	950	950
Glutamine	0	0	100	0	100	0	100

Plate 1. Callus formation on upper surface of leaf (Magnification: 40x)

I). Five growth regulators: 2,4-D, NAA, IBA, Kinetin and BAP were used for callous induction with different levels. 160 explants were tested for each different treatment combinations (seven media and growth regulator combinations).

Callus was induced from spring- and autumn-collected plant material as explant source with light and dark incubation conditions in growth room by four subcultures during four months. Incubation in light includes a 16-h photoperiod at 25ºC. Relationship between percentage of callus production and modified media were statistically analyzed by chi square test by using software SPSS ver. 11.5. Differences between percentage production were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

The effect of different combinations of auxins and cytokinins on callus induction was studied. The effect of rate and type of auxin and cytikinin were very important not only on the percentage of callus production, but also on callus growth. Among the cytokinins analyzed, BAP was more effective than Kinetin. Callus induction was observed in both low and high concentration of auxin. However, cytokinins with concentration higher than 1 mg L^{-1} had a negative effect on callus production and growth. Application of different combinations of BAP and auxins (IBA, 2,4-D and NAA) in different media explored that rate of callusing and its quality is more affected by nitrogen utilized source than type of auxin (Table II).

Results indicated that callus production has important relationship with incubation conditions (light or dark) and explant situation (collected in autumn or spring). The best calli were got from autumn-collected explants in light. In addition, callus formation was observed just on upper surface of leaf mesophill, then callus cleaves the epidermis and continues to grow (Plate 1).

Effects of nitrogen source on callus production. Significantly ($P<0.05$) high callus induction percentage was observed in medium containing half strength MS nitrogen *viz.* U medium (80%) followed by medium V and Y (65 & 95%), respectively. While minimum callusing percentage was depicted in W medium (40%) (Table III). In cultures of many woody species full strength MS salts have inhibitory effect on organized growth, a toxicity that can be reduced by lowering amount of ammonium or total nitrogen (Tsogas & Bouriquet, 1982; Vieitez *et al.*, 1983; Perez-Bermudez & Sommer, 1987; Bonga & Von Aderkas, 1992). Complete elimination of KNO_3 reduces percentage of callus production, however omission of NH_4NO_3 in comparison with MS medium did not show any important effect. Generally tissue culture of conifers and other forest tree species require a combination of both nitrate and ammonium for good growth (Kirby *et al.*, 1987).

In this study, effects of organic nitrogen in callus production indicated that callus production increased by supplementation of basal media (containing half strength MS nitrogen, medium U) with 100 mg L^{-1} glutamine (Table

Table II. Matrix of the callus production rate/callus quality of autumn-collected explants according to tested media (MS, X, Y, Z, W, V, and U) and hormonal treatments

Horm. Treat.	Con. (mg.L^{-1})	MS	X	Y	Z	W	V	U
BAP & IBA	0.1 & 0.5	III	III	III	II	II	III	III
	0.1 & 2	III	III	III	II	II	III	IV
	1 & 5	I	II	-	I	-	I	I
	1 & 10	-	I	-	I	I	I	II
BAP & 2,4-D	0.1 & 0.5	IV	I	IV	II	I	III	V
	0.1 & 2	III	III	III	II	I	III	IV
	1 & 5	I	-	-	-	-	I	-
	1 & 10	-	-	-	-	-	-	-
BAP & NAA	0.1 & 0.5	III	III	III	II	II	III	IV
	0.1 & 2	II	III	II	III	II	III	IV
	1 & 5	I	I	II	I	I	I	-
	1 & 10	-	I	-	-	-	-	-

(-): Lake of callus production, (I): 5-20% callus production, (II): 21-40% callus production, (41-60% callus production, (IV): 61-80% callus production, (V): 81-100% callus production

Table III. Percentage of callus production in MS and 6 Ms modified media

	MS	X	Y	Z	W	V	U
Number of cultured explants	160	160	160	160	160	160	160
Number of induced callus explants	80	86	95	75	64	105	128
Relative percent of callus production	50	54	59	48	40	65	80

II). In conifer tissue cultures, glutamine has shown growth stimulation. Glutamine is also required for cell division and callus proliferation from protoplasts of Douglas-fir (*Pseudotsugga menziessii)* and maritime pine (*Pinus pinaster*) (Kirby *et al.*, 1987). Martel *et al*. (2001) concluded that nitrogen supply as nitrate only depicted no active growth while calli grown on glutamine showed a higher degree of multiplication in jack pine. Western blot analysis indicated that over the 38-day culture period, glutamine synthetase (GS) level was generally greater in samples grown on glutamine suggesting that GS gene expression is up-regulated when glutamine is provided.

Effects of nitrogen source on shoot proliferation. Adventitious buds were induced on about all excised shoot explants of *J. excelsa* on modified MS media (media X and Y) (Plate 2). Generally, there is less total nitrogen in media used for shoot cultures than in those used for callus or suspension culture (Kirby *et al.*, 1987). Although a reduction in the inorganic nitrogen source of MS medium to half strength can increase the callus induction, but the callus produced did not showed any differentiation. However by elimination of NH_4NO_3 from MS medium, both the growth of lateral buds was induced (Plate 2), and adventitious buds were formed from callus (Plate 3) and also directly from lamina (Plate 4). Verhagen and Wann (1989) reported the rate of somatic embryogenesis in cultures of *Picea abies* was high on a medium in which ammonium nitrate had been replaced by glutamine. Shoot formation occurred in embryo

Plate 2. Adventitious buds on modified MS media (media X and Y) (Magnification: 40x)

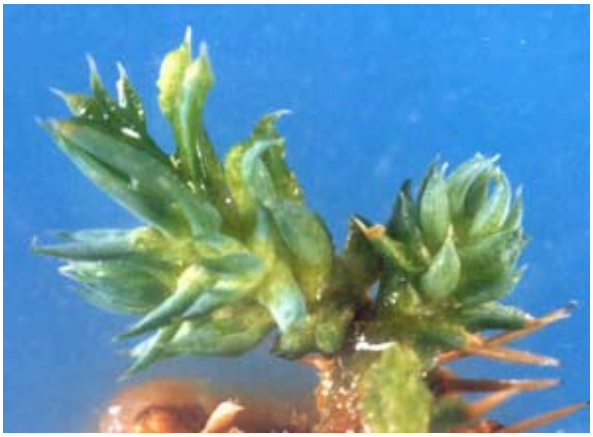
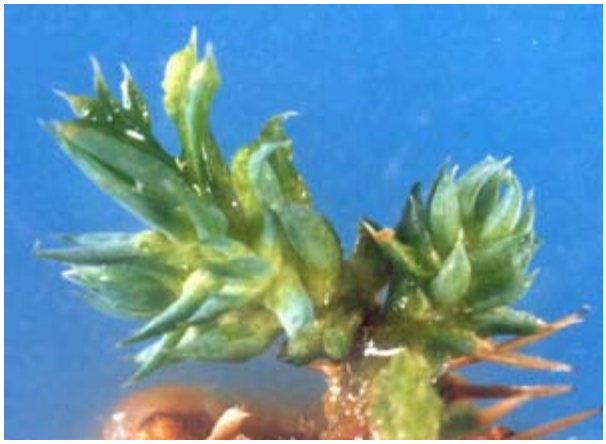


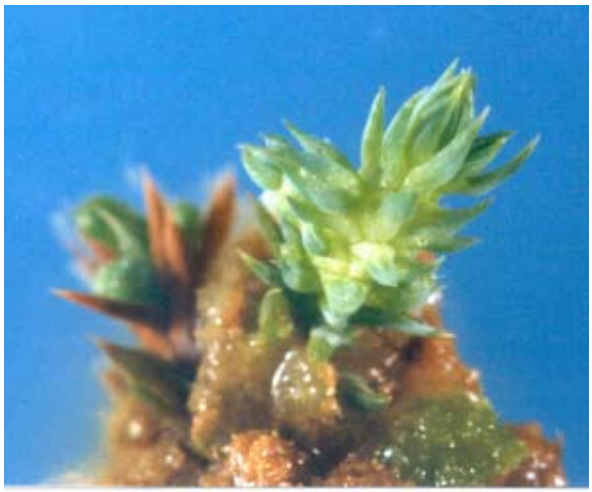
Plate 3. Formation of adventitious buds from callus (Magnification: 40x)



explants of *Pinus strobus* on half strength MS medium but not on full strength MS medium. Shoot formation failed to occur on full strength MS medium because of its high ammonium content (Flinn *et al.*, 1986). However, needle cultures of *Picea sitchensis* showed little organogenesis on media that contained nitrate but no ammonium. With only ammonium in the medium, the needle primordia elongated but no adventitious shoots were formed; the optimal ammonium/nitrate balance for adventitious shoot formation was approximately 1:3 (Thorpe *et al.*, 1989).

Besides, Mashayekhi-Nezamabadi (2003) to examine the role of nitrogen on pH, particularly during the realization

Plate 4. Formation of adventitious buds directly from lamina (Magnification: 40x)



phase of somatic embryogenesis in carrot petiole, used from inorganic reduced and oxidized forms such as $(NH_4)_2SO_4$ and KNO_3 and an organic form of nitrogen in form of casein hydrolysate. According to the results the ammonium sulfate as the reduced form of nitrogen in higher concentrations

reduces pH of the liquid medium to a range of 4 on the pH scale, which leads to the arrest of embryo development. By using potassium nitrate, globular structures gradually developed mature embryos and finally plantlets were formed. Potassium nitrate increased pH of the liquid medium to 7.2. A mixture of different nitrogen forms in the solution is necessary to get suitable results.

It is evident that control of apical dominance exercised by main apex on lateral buds was eliminated. It means the apex does not produce auxins and concentration of auxins at lateral buds is insufficient to inhibit their growth (Baron, 1986). Trewavas (1983) stated the application of nitrate can lead to bud dormancy and release from apical dominance.

In this work, no differences were found in shoot proliferation by supplementation of glutamine in the medium.

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