Somatic Embryogenesis in *Juniperus Procera* using *Juniperus Communis* as a Model

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ABSTRACT

The study of somatic embryogenesis in *Juniperus communis* has been conducted as a preliminary study for the further development of somatic embryogenesis, micropropagation and long-term conservation/cryopreservation in *Juniperus procera*, which is economically and ecologically important and endangered forest species in Ethiopia.

The aim for this particular research was initially an adaptation of optimum half strength lithium chloride-sodium propionate (LP) medium protocol for growth and proliferation of embryogenic culture. But as other conifer species it was difficult to stop proliferation of somatic embryo cell division and bring them to maturation stage. However using optimal concentration of ABA (32 mg/l), it was possible to get maturation response in most of the cultures.

Additional study on the effect of seed extraction to the growing embryogenic culture showed no effect on mature somatic embryos. Rather they were showing faster growth which increases embryogenic mass of each culture. It was also observed that green premature seeds of *Juniperus* are suitable for the initiation and proliferation of somatic embryo.

Key words: Cryopreservation, Juniperus communis, *Juniperus procera*, Somatic embryogenesis.

1. INTRODUCTION

Juniperus procera is an evergreen dioecious, more seldom monoecius tree, which belongs to the family *Cupressaceae*. It is the tallest/largest juniper tree in the world (Pohjonen and Pukkala, 1992; Negash, 1995). It has two developmental phases, the juvenile and the adult stage. They are characterized by their distinctive trunk and crown shapes. It has a pyramidal shape when it is young and more spreading when it gets older. It can reach up to 45 m high when it matures (Berhe and Negash, 1998). The male cones are small and round. They are borne individually and terminally on short branch lets. The female cones are berry-like rounded and, upon ripening, become fleshy and soft (Negash, 1995). Juniper trees flower and give fruits through out the year in Ethiopia without an interrupting resting stage (Achalu, 1995).

J. procera is the only Juniperus species, which is found in the mountains of East Africa and it is one of the two indigenous conifer species found in Ethiopia (Pohjonen and Pukkala, 1992). Locally it is known as Tid and commercially as African Pencil Cedar. It is naturally found in the

central highlands of the country, mainly between altitudes 1800-3200 meters above sea level (von Breitenbach, 1963) with an annual rainfall range that varies between 450 and 1200 millimetres (Jansen, 1981). The optimum altitude range, however, is between 2200 and 2500 meters above sea level (Pohjonen and Pukkala, 1992). It is found in montane vegetation as the dominant species or mixed with other evergreen forests. It grows well in drier climatic zones.

J. procera has been a very important source of wood for timber and fuel. Its wood is fragrant, fine-textured, and strait-grained. It is hardy and resistant to termites and fungal diseases. Because of these distinctive qualities, it is highly valued for the construction of houses, internal structures of churches, furniture and for poles (Achalu, 1995; Negash, 1995). The tree is planted as ornamental and for its shade in homesteads. Its fruits have also some medicinal values for curing headaches, skin diseases etc. Its resin in combination with honey is used as stimulant and as a medicine against ulcers as well as against liver diseases (Jansen, 1981; von Arnold et al., 2005).

Juniperus species have therefore become important in long term rehabilitation projects. The natural habitat of several Juniperus species has been greatly reduced because of overexploitation for timber and fuel wood and clearance for cultivation. Furthermore, several species belonging to the genus Juniperus are hard to propagate via seeds (Ortiz et al., 1998; Helmersson and von Arnold, 2009). The possibility to propagate trees vegetatively creates significant advantages both for the deployment of selected genotypes through mass propagation and for capturing and enhancing genetic gain in breeding programs. Large scale cutting propagation is limited in several coniferous species owing to problems with rooting, aging of mother trees and high costs. Some of these problems can be overcome by using somatic embryos. Somatic embryogenesis has been described in several coniferous species and especially species belonging to the *Pinaceae* family e.g. *Abies*, *Larix*, *Picea* and *Pinus*. However, reports from the *Cupressaceae* family are scarce. Up to now there is only one report showing that it is possible to produce somatic embryos in *Juniperus* (*J. oxycedrus*), however, no mature somatic embryos or plants were obtained. We have chosen common juniper, *Juniperus communis*, as a model species for *J. procera* for studying possibilities and limitations with somatic embryogenesis (Gomez and Segura, 1996; Helmersson and von Arnold, 2009).

This study is carried out on *J. procera*, as a multipurpose tree, because of its use as a good alternative to the exotic species for reforestation and its drought tolerant, easily adaptability and importance for soil and water conservation. Furthermore, the rapid disappearance of this

endogenous and very important tree from its natural habitats is another argument for the development of Somatic embryogenesis.

2. MATERIALS

2.1. Seed samples

200 *J. communis* from Uppsala Genetic Center and 200 embryo seeds of *J. Procera* from Menagesha Suba forest in Ethiopia were use for analysis of this study.

2.2. Culture medium

Half strength lithium chloride-sodium propionate (LP)-medium containing 15 mm NH_4NO_3 (Merck), 30 mm sucrose (BDH), 9.0 µm, 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma) and 4.4 µm N6-benzyladenine (BA) (Sigma), solidified with 0.4% gellan gum (Merck) was used (von Arnold and Eriksson, 1981; Hakan, 1996). *Cryopreservation medium* -5% DSMO were used for short and long term cropyopreservation of *J. Procera*.

3. METHODS

3.1. Seed collection

200 *J. communis* seeds were collected from Uppsala Genetic Center from January to February 2005 and 200 mature, healthy, deep green color and soft look embryo seeds of *J. Procera* were collected from Menagesha Suba forest in Ethiopia from March to April 2004. Seed were collected randomly to get 200 green seeds for initial work of analysis over all.

3.2. Seed sterilization

Premature green seeds were carefully pressed in a mortar by a pistol, and separate seeds from the seed cots and the debris by washing them on a colander under the tap water. Care was taken not to damage the seeds when grinding them by a mortar. Seeds were surface sterilized in 10 % (w/v), sodium hypochloride with two drops of Tween 20 and left on a rotary shaker for 20 minutes to make sure that all seeds are cleaned and washed. The solution was removed and the seeds were washed using 70 % ethanol for 1 minute. Then they were rinsed three times with sterile distilled water and incubated over night in a small amount of sterile distilled water at 4°C.

3.3. Half LP medium preparation

Half LP liquid medium contains 30 mM (10 mg/l) sucrose and 0.44 gm/l filter sterilized L-Glutamine. The pH was adjusted to 5.8±0.01 after adjusting it to the required volume. L-

glutamine was added to the medium after autoclaving the medium on 121° C for 20 minutes under the pressure of 0.3 atm. It is very important to wait until the media cools down to 35-40°C before adding the L-glutamine.

3.4. Mega gametophytes Preparation

Isolation work was done with the help of magnifier placed in a flow hood and by placing the seeds on sterile empty Petri dices. All equipment were sterilized and cooled before every application on the mega gametophyte all work was done under aseptic condition.

Seeds were opened with a scalpel and mega gametophytes were removed carefully with forceps, plated on a Petri dish containing LP half strength media which contain no hormones. 6-10 mega gametophytes were put in one 9 cm Petri dishes, closed tightly by para film or any plastic foil. When there was a doubt for viability, viability test was done before plating them in the growth media. The cultures were incubated at +22°C in the dark. They were sub cultured every three weeks to a freshly prepared 0.5 LP medium.

Mature embryos from seeds of J. procera were inoculated on PGR containing LP medium. It was hard to open each seed and get the mature embryo with out any damage. Taking out the mature embryo procedures are all the same as premature green seeds of *J. Communis*. Sub culturing was done every month.

3.5. Induction of embryogenic cultures

Seeds were removed from the cones and surface sterilized in 70% ethanol (2 min), and 2% sodium hipoclorite (10 min), followed by rinsing three times with sterile water. Explants were dominant and non-dominant zygotic embryos excised from immature seeds under a stereoscope and inoculated in Petri dishes (100 x 15 mm) containing 20 mL of culture medium free of growth regulators (LP_0). The cultures were incubated in the dark at 22 ± 2^0C. After 24 hours magagametophytes were cultured with nine zygotic embryos on freshly prepared media.

3.6. Tracking

Tracking medium used was 0.5 LP followed by 1.2 % (w/v) sea plaque agarose and following by 1.32 gm/l glutamine, after autoclaving the media. Sea plaque agarose is very transparent enabling to see the cell structure under the microscope. The selected well growing cell lines were taken from a suspension culture and left to settle down to the bottom of the jar for a while. Then using a sterile glass pipette and automatic pipette 1.5 ml of liquid half LP medium was sucked to a 6 mm Petri dish and the same amount of cells to the Petri dish and waited until the cell gets in

to the medium. Then to avoid contamination the Petri dishes were sealed by Para film. These were kept in the dark on 22°C for to grow.

3.7. Maturation of somatic embryos

In order to see the morphology of the embryo culture of *J. procera*, different concentration of (ABA) were applied on 15 lines (1, 2, 3, 17, 20, 24, 33, 37, 43, 44, 47, 50, 51, 52, 53) from earlier selection: 16 mg/l, 32 mg/l, 64 mg/l, 128 mg/ and 256 mg/l per one liter of half LP medium followed by 7.5 % (w/v) of polyethylenglycol with (PEG) 4000 (pH 5.8±0.01). Each concentration trial was conducted in three replications.

3.8. Cryopreservation

4gm of the best performing cell line was taken after 14 days of the last subculture and transferred to 10ml half LP liquid medium and pumped carefully. Then the liquid medium was distributed in to 10 cryotubes.

On the first day, the culture in each cryotube was treated by 0.210mL freshly prepared 4M sorbitol ten times over a period of 30 minutes and incubated on a rotary shaker with 100 rpm over night at room temperature in the dark for 24 hours. The same procedure was repeated on the second and third day by adding 0.234mL of 4M sorbitol.

After the third application of 4M sorbitol the cryotubes were placed on ice bath, and then the excessive LP medium was removed. The Tubes were labeled with necessary information and placed them to cryo box which is usable for preservation in liquid nitrogen, at -196^0 C for two weeks. Thawing was beeing done by placing the cryotubes in distilled and sterile water at 45^0 C for 4 minutes, then transferred to 4^oC for one minute. After this, the tubes were transferred to 70% ethanol for one minute, and then they were ready as a liquid medium suspended culture for further use.

4. RESULT

4.1. Isolation and culture of embryos

Both *J. communis* and *J. Procera* seeds were isolated on semi solid LP medium. 145 of *J. communis* and 67 of *J. Procera* seeds showed growth. All these seeds were cultured on fresh LP media every 21 days consequently three times after the first culturing. Growth of both species cell lines was compared. The growth of *J. Communis* was embryogenic, while the growth *J.*

procera was non-embryogenic. Then best performing 53 non-embryogenic cell lines of *J. Procera* were taken for further characterization.

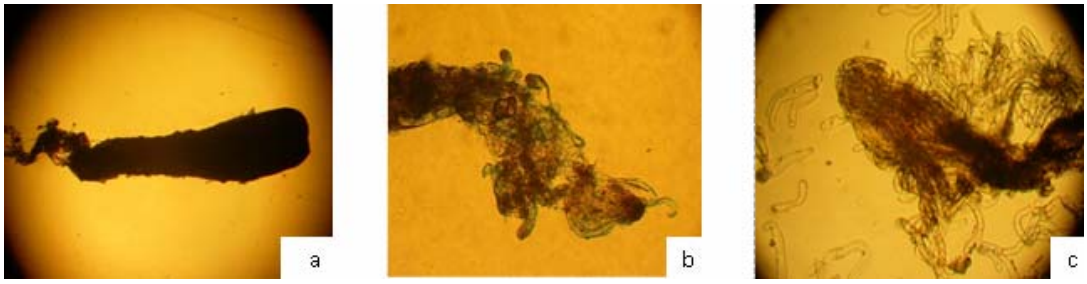

Figure 1. Formation of mega gametophytes of *J. Procera,* a) Full mega gametophyte (b) An embryo going out of the mega gametophyte (c) Several embryos following one big embryo.

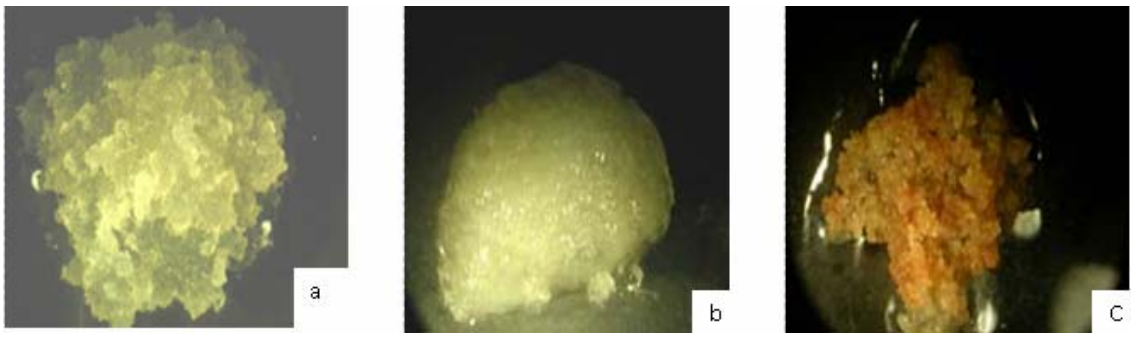

Figure 2. Morphotypes of non- embryogenic cell lines of *J. procera,* (a) Spiky granular (b) smooth (c) cell necrosis

4.2. Characterization of non embryogenic cultures

53 non-embryogenic cell-lines of *J. Procera* grown on semi solid medium differed in embryo morphology and growth habit, and were accordingly be divided into **A**, **B**, **C** and **D** for non-embryogenic morphotypes and **A**, **B** and **C** for growth quality and callus colours, and **–** **(**absence) or **+** **(**presence) for necrotizing cells (Table 1). 53 non embryogenic cell lines gave rise to proliferating non-embryogenic culture (Fig. 1). They were all transparent.

Table 1. Characterization of embryos: morphology and growth habit.

No of lines	Non-embryogenic morphotypes	Growth habit			Selected for cryopreservation
		Growth quality	Callus colour	Necrosis cells	
1.	B	B	B	-	
2.	B	A	B	-	
3.	A	A	A	-	√
4.	C	A	B	-	
5.	C	A	B	-	
6.	D	B	C	+	
7.	C	B	A	-	
8.	C	B	A	-	
9.	D	C	A	-	
10.	D	A	C	+	
11.	B	A	C	+	
12.	D	A	B	-	
13.	D	A	C	+	
14.	C	A	A	-	
15.	D	B	C	+	
16.	C	A	A	-	
17.	A	A	B	-	√
18.	C	A	B	-	
19.	D	C	C	+	
20.	A	A	B	-	√
21.	D	C	A	-	
22.	C	B	B	-	
23.	C	A	A	-	
24.	A	A	A	-	√
25.	C	A	B	+	
26.	D	C	B	+	
27.	C	A	C	+	
28.	C	A	B	+	
29.	C	A	B	+	
30.	B	A	B	+	
31.	D	B	B	+	
32.	D	B	A	+	
33.	B	A	A	-	
34.	D	C	B	+	
35.	A	A	B	+	
36.	D	C	B	+	
37.	B	A	A	-	
38.	D	B	A	-	
39.	C	A	A	-	
40.	D	C	A	-	
41.	D	A	A	-	
42.	A	A	A	+	
43.	B	A	B	-	
44.	A	A	A	-	√
45.	D	A	A	-	
46.	D	A	A	-	
47.	A	A	A	-	√
48.	B	A	A	+	
49.	D	A	A	-	
50.	A	C	A	-	√
51.	A	A	A	-	√
52.	A	A	A	-	√
53.	A	A	A	-	√

Embryogenic morphotype: A = spiky, B = granular/spiky, C = granular, D = smooth, Necrosis cells: + = necrosis, - =no necrosis; Growth quality: A = excellent, B = good, C = no growth; Callus colour: A = White, B = white/yellow, C = brown,

Table 2. Maturation of embryos on ABA for cryopreservation.

No of selected cell lines	ABA concentration	Growth quality	Type of organization in somatic embryos	morphology
3	128	A	A	spiky
	32	B	A	spiky
	64	A	A	spiky
17	128	A	A	spiky
	32	B	A	spiky
	64	A	A	Spiky
20	32	A	A	Spiky
	64	A	A	Spiky
	128	B	A	Granular spiky
24	32	C	B	Granular spiky
	64	C	B	Granular spiky
	128	C	B	Granular spiky
44	32	A	A	spiky
	64	A	A	spiky
	128	B	A	Granular spiky
47	32	A	A	spiky
	64	A	A	spiky
	128	A	A	spiky
50	32	A	B	spiky
	64	A	B	spiky
	128	A	B	spiky
51	32	A	B	spiky
	64	A	B	spiky
	128	A	B	spiky
52	32	A	B	spiky
	64	A	A	spiky
	128	A	A	spiky
53	32	A	A	spiky
	64	A	A	spiky
	128	A	A	spiky

Growth quality: A = excellent, B = good, C = no growth; Callus colour: A = White, B = white/yellow, C = brown, Embryogenic morphotype: A = spiky, B = granular/spiky, C = granular, D = smooth, Necrosis cells: + = necrosis, - =no necrosis

under electro microscope (Fig. 2). Non-embryogenic cultures derived from each mega gametophyte having a specific genotype were given cell line number. There were differences in morphotypes, growth rate, callus colour, and absences/presence of necrosis of cells in the cultures (Table 1). Out of 53 cell lines 15 were defined as spiky or granular/spiky with excellent to good growth quality, white to white/yellow callus color and with out necrosis (Table 1). Spiky cell are cells having the highest potential to differentiate further.

4.3. Adaption for cryopreservation

Out of 15 non-embryogenic fast growing, lacking necrosis cell lines 10 cell lines with best non-embryogenic morphotypes were tested for short and long term cryopreservation. For each line

10 cryotubes were used. Two tubes from each culture were thawed after three weeks. 8 non-embryogenic cell lines showed excellent growth on half LP liquid medium incubated on 22^0C for 24 hours (Table2).

5. DISCUSSION

The morphology of the somatic embryos *J. communis* was similar with in each cell line of *J. procera.*

Somatic embryos of *J. communis* are used as a model system in embryologic/non-embryogenic studies. However, the greatest importance of somatic embryos is its practical application in large scale vegetative propagation. In some cases, somatic embryogenesis is favoured over other methods of vegetative propagation because of the possibility to scale up the propagation by using bioreactors. In addition, in most cases the somatic embryos or the embryogenic cultures can be cryopreserved, which make it possible to establish GeneBank. Embryogenic cultures are also an attractive target for genetic modification (von Arnold et al., 2002).

In order to compare the morphology of the embryogenic culture model of J. *communis* and non-embryogenic culture of *J. procera*, different concentration of ABA were applied on 15 lines of *J. procera.* Both showed the best growth on ABA except the one is embryogenic and the other which was non-embryogenic.

During this work, the only difference was that *J. communis* is embryogenic with much faster growth, while *J. procera* is non-embryogenic with slow growth. That was the reason why 53 lines were performed better growth out of 200 premature green seeds of *J. procera*.

Out of morphologically identified 53 lines only 10 were perceived to grow better on ABA with spiky or granular spiky. These excellent cell lines showed good performance by having white and white- yellowish callus colour and with out necrosis.

Cell lines of *J. procera* with excellent morphotypes and maturation on ABA were used for further propagation and cryopreservation for- long and short- term conservation (Table.2).

Thus, those 10 excellent cell lines were tested for cryopreservation and 8 morphotypes of *J. procera* were showed excellent recovery response on half LP_0 semi solid medium after three weeks cryopreservation at -196^0C liquid nitrogen.

6. CONCLUSION

This study indicated the possible application of tissue culturing technique for conservation of forest tree species. In this particular case, since it is not a problem to get a green premature seeds of *J. procera* all year round, we recommend to use only the premature green seeds for the initiation and proliferation of somatic embryo. By using premature green seeds which gave mass of non-embryogenic cells, it might be possible to bring them to maturation by using the appropriate concentration of ABA, which is going to be useful for micropropagation and further studies on cryopreservation of this species.

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