Micropropagation of Newly Produced F_1 Hybrid of *Eucalyptus* (*E. tereticornis* Sm. X *E. camaldulensis* Dehn. Southern Form)

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Abstract

A new \mathbf{F}_1 interspecific controlled hybrid of *Eucalyptus tereticornis* X *Eucalyptus camaldulensis* (Southern form) was produced during March, 1996 which showed hybrid vigour. Since *de novo* production of \mathbf{F}_1 seed for raising \mathbf{F}_1 plantation is not feasible, cumbersome and labour intensive, thus, an innovative and effective approach to multiply this hybrid via tissue culture was applied to check segregation, retain hybrid vigour and evolve a protocol for mass clonal multiplication.

Key words: Eucalyptus tereticornis, Eucalyptus camaldulensis (Southern and Northern form), hybridization, hybrid vigour, segregation, clonal propagation, tissue culture.

Abbreviations: BAP: benzylamino purine; IBA: indole butyric acid; NAA: naphthalene acetic acid; MS: MURASHIGE and SKOOG medium.

Introduction

Hybridization is a known method to combine the desirable traits of two selected species/parents in F, hybrid and also to capture the benefits of hybrid vigour (heterosis) which is often manifested in certain parent combinations of hybrids. The hunch to test the effect of using "southern form" of E. camaldulensis on the degree of heterosis in combination with E. tereticornis was tried to produce a new hybrid which became creative in yielding fruitful results in these studies. Because earlier in producing FRI-4 and FRI-5 reciprocal hybrids "northern form" of *E. camaldulensis* was used in combination with *E.* tereticornis which displayed a very high degree of sustained and pronounced degree of hybrid vigour both in height and diameter (Venkatesh and Sharma, 1977a, b and c, 1978, 1979) showing superiority in wood volume than the parent species. This new hybrid also displayed hybrid vigour in growth parameters substantially more even than FRI-4 and FRI-5 and the parental species at nursery stage.

The other main aim of using "southern form" of *E. camaldulensis* was to capture the expression of the desirable traits present in it for frost resistance and better response to a gradual hardening to low temperature (PRYOR and BRYNE, 1969; AWE and SHEPHARD, 1975).

Since a very limited quantity of \mathbf{F}_1 seed was available from this cross and de novo production of hybrid seed is cumbersome, cost and labour intensive, thus, an innovative and effective approach of multiplying \mathbf{F}_1 hybrid through tissue culture (micropropagation) was adopted. The experiments were carried out also keeping in view to find a solution to check problems of the expression of a spectrum of segregating \mathbf{F}_2 population having more trees with inferior and less number of trees with superior traits, as the parents are heterozygous due to their cross pollinating nature prevalent in the species.

Material and Methods

Controlled crossing

In Australia originally there occur two forms of Eucalyptus camaldulensis viz Northern and Southern which are confined

to Northern and Southern region of Australia. The two forms can be distinctly characterized from each other based on operculum shape, juvenile leaves, bark colour, presence or absence of lignotubers and branching habit (PRYOR and BRYNE, 1969). One of the ramet of *E. tereticornis* established in a hedge garden from Candidate Plus Tree (CPT) material initiated flowering during March, 1996. Taking advantage of low height of the plant, 56 flower buds were emasculated with the help of sterilized surgical blade. The emasculated flowers were crossed using the pollen collected from a tree of E. camaldulensis (Southern form) introduced from Australia during early 1972 growing at New Forest Campus of FRI, Dehradun (altitude 610 m, latitude 30 °N, Longitude 78 °E, annual rain fall 216 cm), when stigma became receptive. Thirteen capsules were harvested as a result of crossing, out of which, from the chaff extracted 80 healthy seeds.

Explant

A sample of nine seeds out of the aforementioned F_1 seeds was taken, washed in liquid detergent (Teepol 5 to 10 drops/100 ml), and then surface sterilized in 20% sodium hypochlorite solution for 30 minutes. After sterilization to remove the sterilant, seeds were washed well (five to six times) in sterilized distilled water, and then inoculated on MS medium (devoid of growth regulators) for germination under aseptic conditions. Nodal segments measuring 5 mm to 8 mm were taken from aseptically germinated seedlings after 20 days of inoculation.

Culture medium

For induction of morphogenesis, proliferation and rooting, MS medium (Murashige and Skoog, 1962) having 3% sucrose (as a carbohydrate source) was used. The medium was gelled with 0.7% bacteriological agar (Ranbaxy). The pH of medium was adjusted to 5.8 by using 1 N NaOH or 1N HCl prior to adding agar. The culture medium was autoclaved at 121°C and 1.0 kg/cm^2 pressure for 15 minutes.

For multiplication of the cultures, combinations of BAP (1.0~mg/l~and~2.0~mg/l) with NAA (0.01~mg/l~and~1.0~mg/l) were selected (Table~1). In vitro grown shoots measuring 1.0 cm to 1.5 cm were harvested and kept on 1/2 strength MS medium supplemented with different concentrations of IBA (0.1~mg/l) to 1.0 mg/l) and NAA (0.5~mg/l) alone and in combination for rooting (Table~2).

Culture conditions

All the cultures for proliferation and rooting were maintained and incubated at 25 ± 1 °C for 16 hours in light (illuminated by 40 watt fluorescent tubes, 1200 lux) and for 8 hours in dark.

Acclimatization

Rooted shoots after attaining a height of 3.0 cm to 4.0 cm, were transferred to pots containing a mixture of soil: sand: manure (1:1:1) and covered with perforated polythene bags. Holes were made in the polythene bags for gaseous exchange.

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 $\it Table~1.-Effect~of~BAP~and~NAA~on~multiple~shoot~formation~and~elongation~of~shoots.$

C ₁	2.0 1.0 1.0 2.0 1.0 2.0 1.0 1.0	NAA 1,0 1,0 0.01 1,0 0.01	60 days 32.4±0.73 49.6±1.58 37.6±0.82 12.4±0.83 32.8±0.77 27.0±1.01	90 days 65,2±1,58 95,6±1,66 84,6±1,54 25,0±1,65 52,4+0,83	120 days 134,0±5,18 222,0±7,29 149,6±3,30	120 days	135 days	150 days	180 days
C ₂	1.0 1.0 2.0 1.0 1.0	1,0 0.01 1,0 1,0 0.01	49.6±1.58 37.6±0.82 12.4±0.83 32.8±0.77	65,2±1,58 95,6±1,66 84,6±1,54 25,0±1,65	134,0±5,18 222,0±7,29 149,6±3,30	+++		++++	
	1,0 2,0 1,0 1,0	0.01 1.0 1.0 0.01	37.6±0.82 12.4±0.83 32.8±0.77	84.6±1,54 25.0±1,65	149,6 <u>+</u> 3,30				
	2.0 1.0 1.0	1.0 1.0 0.01	12.4±0.83 32.8±0.77	25.0±1.65			j :		1
	1.0 1.0	1.0 0.01	32.8±0.77			Ì		<u> </u> -	
C ₃	2.0	0.01		52 440 62	75.2±1.58		++	++	++++
C ₃	2.0		27.0 <u>+</u> 1.01	JZ.4TU.63	104.4±1.46				ł
C ₃		1.0	I	47.0 <u>+</u> 85.0	95,2 <u>+</u> 1.58				
1	1.0	1.0	32.0 <u>+</u> 0.85	64.6±2.29	135.6 <u>+</u> 3.21	+++	++++	++++	++++
		1.0	45.6±1.35	95.2 <u>+</u> 1.58	220.4±7,57				
	1,0	0.01	36 . 8±0 . 95	68,6 <u>+</u> 2,96	140.0 <u>+</u> 3.16				
C ₄	2.0	1,0	7.0±0.40	23.0 <u>+</u> 0.85	44.8+1.66		+	++	++++
1	1,0	1.0	17 .6<u>+</u>0.8 3	46.6 <u>+</u> 1.69	90.6 <u>+</u> 3.39				
	1.0	0.01	12.4 <u>+</u> 0.92	26,8±0,87	52.2 <u>+</u> 0.91				
C ₅	2.0	1,0	1,2±0,18	3.60 <u>+</u> 0,36	11,6 <u>+</u> 2,38			+	++++
1	1.0	1.0	3.8 <u>+</u> 0.44	11.0 <u>+</u> 0.4	34 . 4 <u>+</u> 1.51				
	1.0	0.01	1.5 <u>+</u> 0.22	7,8 <u>+</u> 0,87	17.8 <u>+</u> 0.77				
C ₆	2,0	1,0	2,4 <u>+</u> 0,22	12,4 <u>+</u> 0,83	25.0±1.65	+	++++	++++	++++
	1.0	1.0	44.6 <u>+</u> 1.54	86,0 <u>+</u> 1,6	200,0 <u>+</u> 6,32				
	1.0	0.01	3,6 <u>+</u> 0,22	17.2 <u>+</u> 0,77	27 . 6 <u>+</u> 2.24				
C ₇	2.0	1.0	2.4±0.22	17.6 <u>+</u> 0.83	35.6 <u>+</u> 1.61			+	+++
	1.0	1.0	22.4 <u>+</u> 0.83	52.6±1.15	132.0±8.07				
	1.0	0.01	3,8 <u>+</u> 0,33	25.4±1.54	62.6 <u>+</u> 3.19				
C ₈	2.0	1.0	2,6+0,22	18.4 <u>+</u> 0.46	31.2±1.25			+	+++
	1.0	1.0	13.0±0.8	38.4 <u>+</u> 0.83	82.8 <u>+</u> 3.27				
	1.0	10.0	4.6 <u>+</u> 0.21	21,8±1,15	32.4 <u>+</u> 0.73				
C ₉	2.0	1.0	1.6+0.22	5.4+0.22	23.2±0.87		+	+++	++++
	1.0	1.0	8.2 <u>+</u> 0,77	27.6 <u>+</u> 0.73	56.2±1.66				
	1.0	0,01	3.4 <u>+</u> 0.22	8,8 <u>+</u> 0,34	25.0 <u>+</u> 1.83				

⁻ Nil, + > 8 mm to 10 mm, ++ > 10 mm to 15 mm, +++ > 15 mm to 20 mm, ++++ > 20 mm to 35 mm, \pm SE

The polythene bags were withdrawn after 7 days when a new pair of leaves started emerging on the shoots.

For calculating the multiplication potential of this hybrid on different media combinations 24 replicates of each clone were maintained for each treatment. Subculturing was done periodically at an interval of 30 days. For this a cluster of buds (4 to 5 buds) was separated with the help of scalpel and transferred to the same nutrient medium on which nodal explants were inoculated. After 120 days when multiplication rate was more or less uniform, MS medium having 1.0 mg/l BAP was used for further subculturing of shoots. For rooting experiments, 25 replicates of each clone were maintained for each treatment.

Results

All the 9 seeds germinated in MS medium. As seeds belonged to a controlled F_1 hybrid, thus, each seed was considered as a separate genotype owing to the heterozygous nature of the parental species. Accordingly, all 9 genotypes $(C_1 \ \text{to} \ C_9)$ were established under uniform conditions. Cultures produced from single seed were considered as a separate clone and in this manner, all nine clones were established $(C_1 \ \text{to} \ C_9)$ for mass multiplication.

(i) Shoot bud initiation, multiplication and elongation

Initiation of shoot buds started without an intervening callus phase within 15 days from the date of inoculation of the nodal explants. All the clones/cultures responded to different media compositions tried for multiplication and elongation but in a different manner ($Table\ 1$). Maximum number of shoot buds per culture was observed in MS medium supplemented with equal quantity of BAP 1.0 mg/l + NAA 1.0 mg/l which was followed by 1.0 mg/l BAP + 0.01 mg/l NAA and 2.0 mg/l BAP + 1.0 mg/l NAA respectively ($Table\ 1$, $Figure\ 1$). After 120 days stable cultures could be maintained on MS medium having only 1 mg/l BAP as a growth regulator.

Within a period of 120 days, only cultures of two clones viz C_1 and C_3 exhibited multiplication and elongation of shoots while in the remaining (C_2 and C_4 to C_9) proliferation was observed but no elongation at all and shoots were very small in size (< 5 mm), however, after 150 days all the cultures of the clones (C_1 to C_9) achieved more or less same elongation of proliferated shoots (Table 1).

(ii) Rooting

Response to rooting of C_1 to C_9 was different in different concentrations of hormones up to 180 days (Table 2). Clone

 $\it Table~2.-Effect~of~different~concentrations~of~auxins~on~rooting.$

	PGR augmen		Percentage of shoots rooted after					
Clone	IBA	NAA	120 days	135 days	150 days	180days		
C ₁	0.1	0	50	60	65	70		
	0.5	0	80	80	85	90		
	1.0	0	50	70	85	98		
	0.5	0.5	75	75	80	80		
C ₂	0.1	0	-	30	50	60		
	0.5	0	-	45	70	75		
	1,0	0	_	40	75	88		
	0.5	0.5		50	60	70		
C ₃	0,1	0	50	60	65	72		
	0.5	0	78	80	85	90		
	1.0	0	48	70	85	99		
	0,5	0.5	75	77	82	82		
C ₄	0,1	0	-	20	25	50		
	0.5	0	_	25	30	70		
	1,0	0	-	30	40	85		
	0,5	0.5	_	22	25	48		
C ₅	0,1	0	-	_	19	40		
	0.5	0	-	-	25	65		
	1.0	0	_	_	30	80		
	0,5	0,5	_	_	20	50		
C ₆	0.1	0	18	30	50	60		
	0.5	0	25	40	55	70		
	1.0	0	28	50	70	85		
	0.5	0,5	20	35	50	70		
\mathbf{C}_{7}	0,1	0	-		20	40		
	0.5	0	-	-	25	60		
	1.0	0	-	_	30	70		
	0.5	0.5	-	-	25	55		
C ₈	0 ,1	0	-	_	20	40		
	0.5	0	_	_	24	60		
	1,0	0		_	32	75		
	0.5	0.5	-		25	60		
C ₉	0.1	0	_	22	40	65		
	0.5	0	_	25	50	72		
	1,0	0	_	35	70	88		
	0.5	0,5	-	24	58	70		

⁻ Shows no elongation hence could not be taken for rooting.

number $\mathrm{C_1}$ and $\mathrm{C_3}$ had shown better response over the other clones, but after 180 days little variation in respect of rooting percentage was observed among different clones. The roots were thin like a thread in 0.1 mg/l IBA while in 0.5 mg/l IBA + 0.5 mg/l NAA little callusing was observed at the junction of shoot and root. Highest rooting percentage in all clones was achieved in 1 mg/l IBA followed by 0.5 mg/l IBA (Figures 2a and b). Initially the cultures took 14 to 15 days for rooting, but subsequently, after repeated subculturing (6 to 7 times) rooting took only 5 to 7 days.

(iii) Acclimatization

After transfer of plantlets to soil in pots 90% success in survival rate was observed (Figure 3). The tissue culture plants, after attaining a height of 70 cm to 80 cm, were outplanted in the field for establishing a hedge garden at New Forest, Dehradun as well as at Haryana for laying out a field trial for comparison and production of more improved planting material along with other selected clones.

Discussion

In the present investigation C_1 to C_9 genotypes responded differentially referring to the rate of multiplication, elongation (Table 1) and rooting (Table 2) to a certain stage of development (120 days). But later on after 180 days all of them adopt-

Figure 1. - Axillary shoot proliferation.

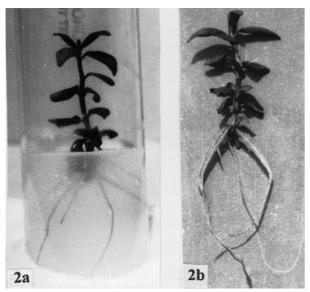


Figure 2. – Rooting of microshoots. a – In vitro rooting. b – Rooted plantlet ready for transfer to polypots.

ed more or less a uniform elongation pattern. These results are in agreement with the work carried out by Das and MITRA (1990) using seeds of $Eucalyptus\ tereticornis$ from 50 individual trees. The differential response to the above parameters observed in the case of C_1 to C_9 genotypes could be attributed to the different genotypic constitution of each seed as the maternal and paternal parents were basically heterozygous.

The potential for shoot elongation in C_1 to C_9 genotypes increased after 6 to 7 subculturing which resulted to intense axillary budding, contrary to work carried out by Chauhan et al. (1996) on in vitro propagation of Eucalyptus hybrid FRI-4 (E. tereticornis Sm. x E. camaldulensis Dehn. northern form) in which they observed reduction in potential for shoot elongation after 5 to 6 subcultures. Similarly, in the initial cultures rooting took 14 to 15 days but subsequently after 6 to 7 subculturing rooting took only 5 to 7 days. It would be worthwhile to mention here that subculturing process may change the physiological state of the explant. However, in the present study as the explant material (seed) was already juvenile, improved propagation and rooting behaviour following increase number of subculturing seems to be caused by more or less expressed adaptation capability of the nine genotypes to the culture conditions. There exist reports of rejuvenation of mature tissue after subculturing. Gupta et al. (1980), while working on 100-year-old tree of teak (Tectona grandis) reported that rooting percentage increased from 10% in primary culture to 60% in the second and subsequent subcultures. Further, in bud cultures of 20-year-old trees of Eucalyptus citriodora (GUPTA et al., 1981) none of the stage three treatment (rooting experiments) resulted in the root formation in either the initial explant or in the first three subcultures. However, 35% to 40% of shoots rooted at fourth subculture and the percentage of rooting further increased 45% to 50% in fifth and subsequent passages of subculture. These reports clearly indicate that subculturing may modify the physiological state of explant and results in return of some characteristics to juvenility which confirm to our observations in the case of CPT's selected out at the age of 18 to 26 years in the case of Eucalyptus tereticornis (KAPOOR et al., in press).

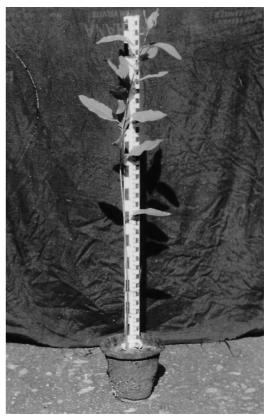


Figure 3. - A two month old potted plant.

The use of "southern form" in this crossing work has given rise to a new hybrid having potential for better growth and resistance to frost at nursery stage, however, its performance will also be tested for growth, resistance to frost and its adaptability in field, for which trials have already been established in the state of Haryana along with local and other selected clones raised through macropropagation (by rooting of cuttings) for comparison. Hybrid clones have also been established in hedge garden at New Forest, Dehradun, for producing more \mathbf{F}_1 hybrid planting stock for further testing in field at different locations.

This package of technology has the potential for high productivity, uniform population on mass scale and checking the problem of segregation, which is commonly met when plants are raised through seeds collected from \mathbf{F}_1 hybrid and the population so raised show more inferior phenotypes than superior ones which is not acceptable to farmers.

Since no loss of multiplication potential of this new hybrid was observed in more than one year of four-weekly subcultures, production of more than a million plantlets from a single explant in a year is feasible by the use of this technology. If *in vitro* technology is combined with macropropagation (rooting of cuttings) of tissue culture plants it will reduce the production cost drastically.

Tissue culture techniques showed a high multiplication rate of this new \mathbf{F}_1 hybrid. Therefore, this *in vitro* protocol developed for mass multiplication of \mathbf{F}_1 hybrid assumes economic importance due to (i) retention of hybrid vigour (ii) capable to leading improved productivity/ha/year, (iii) uniformity in the plantations and (iv) high multiplication rate from meagre quantity of rare \mathbf{F}_1 hybrid seed produced after difficult and intensively time consuming efforts.

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Buchbesprechung

Der Zugang zu genetischen Ressourcen nach dem Übereinkommen über die biologische Vielfalt und dem deutschen Recht. Reihe: Umweltbundesamt Berichte 7/96. Von R. Wolfrum und P.-T. Stoll. 1996. Erich Schmidt Verlag, Berlin, Bielefeld, München. ISBN 3-503-04039-0. 134 Seiten. Kartoniert DM 38,—.

An English version of this book is also available under the title "Access to Genetic Resources under the Convention on Biological Diversity and the Law of the Federal Republic of Germany". Berichte des Umweltbundesamtes, Nr. 8/1996. 1996. Erich Schmidt-Verlag, Berlin, Bielefeld, München. ISBN 3-503-04040-4.

Am 21. März 1994 ist für die Bundesrepublik Deutschland das "Übereinkommen über die biologische Vielfalt" (Convention on Biological Diversity) in Kraft getreten, das im Rahmen der Konferenz der Vereinten Nationen für Umwelt und Entwicklung im Juni 1992 in Rio de Janeiro verabschiedet wurde. Artikel 15 des Übereinkommens behandelt den Zugang zu genetischen Ressourcen in Anbetracht der souveränen Rechte der Staaten in bezug auf ihre natürlichen Ressourcen. Die Befugnis, den Zugang zu genetischen Ressourcen zu bestimmen, liegt bei den Regierungen der einzelnen Staaten und unterliegt den innerstaatlichen Rechtsvorschriften. Ziel der vorliegenden Abhandlung war es, Artikel 15ff. daraufhin zu prüfen, wo sich nach deutschem Recht Ansatzpunkte bzw. rechtliche Grenzen ergeben. Einleitend wird das Übereinkommen hinsichtlich einer nachhaltigen Nutzung der Ressourcen, der Kommerzialisierung und insbesondere hinsichtlich seines juristischen Anwendungsbereichs und des politischen und rechtlichen Umfelds untersucht. Sodann werden in einem 1. Teil Begriffe und Grundfragen der Zugangsregelung analysiert. Dabei kommen die Autoren zu der Auslegung, dass das Übereinkommen

den einzelnen Staaten die Zuständigkeit zur Regelung des Zugangs zu Ressourcen zuweist, soweit diese Ressourcen in ihrem Hoheitsgebiet liegen und dort ihren Ursprung haben bzw. von dem betreffenden Staat rechtmäßig erworben worden sind. In einem 2. Teil wird die Gestaltung des Zugangs zu den eigenen Ressourcen der Bundesrepublik Deutschland erörtert. Die Autoren kommen zu dem Ergebnis, dass das Übereinkommen den Mitgliedstaaten große Gestaltungsspielräume bei den rechtlichen Regelungen über den Zugang zu den Ressourcen belässt. In einem 3. Teil werden schließlich die Möglichkeiten zum Vollzug und zur Durchsetzung des aufgrund des Übereinkommens zu schaffenden internationalen Zugangssystems aufgezeigt. Auf die schwierige Überschaubarkeit und Kontrollmöglichkeit bei dem Verkehr mit genetischem Material wird hingewiesen. Das bedeutet, dass der einzelne Staat den effektiven Vollzug der Zugangsregelung nicht gewährleisten kann. Daher müssen alle Staaten gemeinsame Maßnahmen ergreifen, um die weitere Verfügung oder den Gebrauch vor allem von unzulässig erworbenen genetischen Ressourcen einzuschränken. In einem abschließenden 4. Teil werden die Teilhabeordnung des Übereinkommens und ihre Verschränkung mit der Zugangsregelung behandelt. Hierbei geht es insbesondere um die Forschungsbeteiligung, den Technologietransfer, die Beteiligung an gewonnenen Ergebnissen und an eine sog. Repatriierung von Informationen. Die Autoren stellen aber klar, dass vor allem hinsichtlich der Teilhabe die Austauschordnung nach dem Übereinkommen noch eine rahmensetzende Konkretisierung braucht. Die Abhandlung ist eine wichtige und empfehlenswerte Informationsquelle für alle, die sich ganz allgemein mit genetischem Material befassen und sich der Insitu- und Ex-situ-Erhaltung von in- und ausländischen genetischen Ressourcen widmen.

B. R. Stephan (Grosshansdorf)

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