STANDARDIZATION OF *Punica granatum* EXPLANT AND CALLUS INDUCTION THROUGH MICROPROPAGATION - INDIRECT ORGANOGENESIS

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Abstract

Pomegranate (Punica granatum L.) and variety name 'Bhagwa' is an ancient, important fruit crop in India and in subtropical countries of the world as it possess various pharmaceutical and therapeutic properties. This is subjected to bacterial blight caused by Xanthomonas axonopodis pv causing a huge loss of about 50-100% in production. In order to develop a disease resistant pomegranate variety, micro propagation is necessary. The different explants such as leaves, nodes, apical shoot and petals were selected. The explants were passed through surface sterilization process and found that the mortality rate was least with the apical shoots as explants when compared to other explants. Callus Initiation was done with several treatments and the percentage of callus growth was identified using one way ANOVA by which variance was tested using Fischer's F test and LS (Least Squares) means by Duncan's multiple range test which proved that the LS means was higher for all the explants those undergone MS + Sucrose (30g/l) + Adenine sulfate – 40mg/l + 6BAP – 5 mg/l treatment, specifically apical shoot explants showed 92% callus growth than other explants. The elimination of polyphenol exudation was successful with silver nitrate of 5 mg/l which eradicated the browning of the tissues and paved way for the regeneration of the shoots.

Key Words: *Bhagwa, Micropropagation, Apical shoots, Callus induction, ANOVA, Duncan's test, Polyphenol exudation*

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1. INTRODUCTION

Pomegranate, 'Bhagwa' is a shrub, traditionally propagated by seeds and cuttings resulting in a highly variable progeny. It is being planted widely in various parts of India. Each variety is known for its specific quality giving high yield. 'Bhagwa' pomegranate is the common commercial fruit crop in Maharashtra with bigger fruit size, colored thick skin which is an important reason for its market [1]. This variety is recently getting more demand in market and production because of wide spreading disease bacterial blight caused by *Xanthomonas axonopodis* [13].

Abundant varieties of Pomegranate such as Mridula, Bhagwa, Ruby, Arakta and Ganesh which are commercially grown crops. The superiority to the fruit yield was shown by Jalore Seedless, G 137 and Ganesh. The Mridula beat all the varieties in its Quality. These varieties excelled in Rajasthan and Punjab [12]. A variety named Kandhari proved the highest Total Soluble Solids (TSS) and Total sugars which is very common in Himachal Pradesh. Pomegranate also possesses Ornamental values [6]. Its main occurrence is as Double Flower, Dwarf or Nana. It is widely cultivated as decorative fruit tree in China

Maximum cultivation of pomegranate is in states of Maharashtra and North Western Karnataka which are very close to the western port of Mumbai for exporting to Gulf and European countries [3]. 'Bhagwa' variety has high acceptance in European market. Till 2012, the export of Pomegranate from India was in tons and the profit was in lakhs [2].

The Pomegranate also possesses various medicinal values. It fights against Breast Cancer, Prostate Cancer, Lowers Cholesterol, Maternal consumption of Pomegranate also cures Brain damage, regular intake of pomegranate juice decreases the risk of heart stroke, gives immunity [11]. Each part of the plant has several health benefits [4]. The rind of the pomegranate when ground into powder and diluted with oil cures anal itching. Such a juicy medicinal tree is being severely attacked by the disease and pests recently attacking a single plant which is easily being spread to the whole crop in few days [5].

This study was intended to standardize the pomegranate explant and to achieve callus induction through indirect organogenesis and the location selected was Hosur near Bangalore which is Bacterial blight affected area.

2. MATERIALS AND METHODS

2.1 STUDY LOCATION

The research work was conducted in the Tissue Culture Laboratory Division, Genewin Biotech, Hosur, Tamil Nadu. The Laboratory Center is located 48 km away from Bangalore at 12.7200° N latitude and 77.8200° E longitude.

2.2 GENETIC MATERIALS

Ten pomegranate healthy plants of 'Bhagwa' were obtained from Vijayalakshmi Nursery, Shimoga, Shimoga district, Karnataka, India for allotting the master code throughout the study and also to establish the mother plant pedigree of 'Bhagwa' Pomegranate. 'Bhagwa' variety is known for the rich coloured fruit which is getting invisible in the market as it is highly prone to Bacterial Blight [7].

2.3 STOCK PLANTS ESTABLISHMENT

In the Laboratory Green House, Mother Plant Pedigree had been established from the obtained plants. The sacks were named as PM1, PM2, PM3, PM4, PM5, PM6, PM7, PM8, PM9, and PM10. Pretreatment of obtained plant root zone was done by soaking the cuttings in 0.2% Streptomycin as bacterial treatment for 2 minutes and rinsed in distilled water; 0.2% Carbendazim as fungal treatment for 2 minutes and thoroughly rinsed in distilled water. The treated cuttings were planted in a mixture of soil, sand and coco peat (1:1:1) [8]. These stock plants were maintained in the greenhouse at an average temperature of 35- 40°C.

2.4 EXPLANTS PREPARATION

The explants such as nodes, apical shoot tips, leaves, petals were collected from six months old stock plants grown in the green house. The collected explants were brought to the production laboratory and washed thoroughly in running tap water for 10 min. in order to eliminate the muddy particles from the explants. Nodal explants were excised of about 1 – 1.5 cm with the help of secateur. Apical shoot tips, leaves and petals were excised of about 1 cm with the help of forceps and scalpel [14].

2.5 EXPLANTS STERILIZATION

The explants such as nodes, leaves, apical shoot tips and petals were soaked in antifungal and antibacterial solution containing carbendazim (0.1%) and streptocycline (0.1%) for 30 minutes. The soaked explants were removed and treated with antiseptic solution Dettol or Savlon (5ml/l) followed by washing the explants in sterile water. The sterilization is followed by the treatment of detergent, Polysorbate 20 for 15 minutes. The explants were washed with sterile water three times to ensure the complete wash of detergent.

The sterilization is further carried out inside laminar air flow chamber; explants were treated with Ethanol (70%) for 30 seconds and Mercuric chloride (0.1%) for 5 min. The explants were removed and washed with sterile water 3 times to eliminate the toxic effects of Mercuric chloride.

The Mortality rate was calculated by

$$\% \text{ Mortality} = \frac{\text{Explants contaminated}}{\text{Total no of Explants}} \times 100$$

2.6 EXPLANTS INITIATION

The sterilized explants such as nodes, leaves, apical shoot tips and petals were inoculated in following MS basal media treatments + Sucrose 3% with various growth regulator concentrations for callus induction.

T0: Control
T1: Adenine sulfate – 40mg/l + 6BAP – 5 mg/l
T2: Adenine sulfate – 40mg/l + 6BAP – 1mg/l
T3: Adenine sulfate – 40mg/l + 6BAP – 2 mg/l
T4: Adenine sulfate – 40mg/l + 6BAP – 3 mg/l
T5: Adenine sulfate – 40mg/l + 6BAP – 4 mg/l
T6: NAA – 1 mg/l + 6BAP – 4 mg/l
T7: NAA – 2 mg/l + 6BAP – 4 mg/l
T8: NAA – 3 mg/l + 6BAP – 4mg/l
T9: NAA – 4 mg/l + 6BAP – 4 mg/l
T10: NAA – 5 mg/l + 6BAP – 4 mg/l

The explants such as Nodes and Apical shoots were placed in upright position on each treatment; the Leaves explants were placed abaxial side down on each treatment; each treatment consisted of 40 jars containing two explants in a single jar [9]. The same was repeated for other explants. The inoculated jars were incubated.

2.6.1CULTURE CONDITIONS

For one week, inoculated explants are kept under dark. Then they are subjected under light intensity in the growth room were 10-12 h. photoperiod provided by cool white fluorescent lamps of 1500-3000 lux, temperature of about 25 ± 2 °C and humidity of 35 - 40%. The observation for the callus induction was recorded after 4-5 weeks.

2.7POLYPHENOL EXUDATION

The most common problem in pomegranate explants is the interference of the polyphenols exudations in growth and becomes toxic for the plant itself [10]. It has the capability to turn the media into dark brown causing slow death of the explants. For the elimination of the polyphenols, the treatment consisted of MS basal media treatments + Sucrose 3% + Adenine sulfate – 40mg/l + 6BAP – 5 mg/l with the following polyophenol exudators.

Table – 1: Trial Treatments for Polyphenol Exudation

TRIALS	ASCORBIC ACID + CITRIC ACID (mg/l)	**SILVER NITRATE (mg/l)**
1	25 + 12.5	1
2		2
3		3
4		4
5		5
TRIALS	Ascorbic Acid + Citric Acid (mg/l)	**PVP (mg/l)**
6	25 + 12.5	100
7		200
8		300
9		400
10		500
TRIALS	Ascorbic Acid + Citric Acid (mg/l)	**ACTIVATED CHARCOAL (mg/l)**
11	25 + 12.5	100
12		200
13		300
14		400
15		500

The trial treatments were further performed on the apical bud explants as it responded well with the callus initiation treatment consisted of MS basal media treatments + Sucrose 3% + Adenine sulfate – 40mg/l + 6BAP – 5 mg/l, and was incubated in the growth room.

2.7.1CULTURE CONDITIONS

The culture conditions maintained in the growth room were 10-12 h. photoperiod provided by cool white fluorescent lamps of 1500-3000 lux, temperature of about 25 ± 2 °C and humidity of 35 - 40%. The observation for the callus induction was recorded after 4-5 weeks.

3. RESULTS AND DISCUSSION

3.1 Effect of Surface Sterilization on Explants

The results of surface sterilization on explants were recorded after a week and the mortality rate of the explants were compared.

Table - 2: Effect of Surface Sterilization on Explants

EXPLANTS	TOTAL NUMER OF EXPLANTS	GOOD	CONTAMIN ATION
Nodes	50	30	20
Leaves	50	15	35
Apical Shoot	50	48	2
Petal	50	18	32

Nodes – 40%
Apical shoots – 4%
Leaves – 70%
Petals – 64%

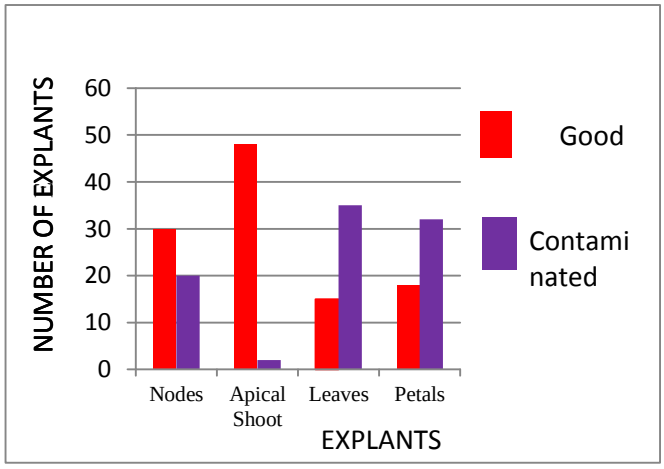

Chart - 1: Effect of Sterilization on Explants

The mortality rate was higher when leaves and petals were used as explants; Sterilization was effective with apical shoots as explants and moderately effective for the nodal explants.

3.1 CALLUS INDUCTION ON EXPLANTS:

The incubated explants undergone for analysis by Fischer's test broadening the variance and Least Squares means by Duncan's Multiple Range test which concluded the best Treatment suitable for the explants.

Table - 3: Analysis of variance in nodal explants

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	10	35272.294	3527.229	2635.561	
Error	98	131.156	1.338		
Corrected Total	108	35403.450			<0.0001

Table - 4: Analysis of variance in apical shoot explants

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	10	46589.431	4658.943	2278.073	
Error	98	200.422	2.045		
Corrected Total	108	46789.853			<0.0001

Table - 5: Analysis of variance in leaves explants

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	10	18596.833	1859.683	444.956	
Error	98	409.589	4.179		
Corrected Total	108	19006.422			<0.0001

Table - 6: Analysis of variance in petal explants

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	10	19132.659	1913.266	68.616	
Error	98	2732.589	27.884		
Corrected Total	108	21865.248			<0.0001

The test used here is the Fisher's F test for the analysis of variance using one way ANOVA in all explants. The probability corresponding to the F value in this case was 0.001 which indicates that there is significant difference in the growth of callus with respect to all treatments compared against control; it means that we would take a 0.1% risk to conclude that the null hypothesis (no effect of the treatment) is wrong.

Table - 7: Analysis of difference between treatments by Duncan's multiple range test – Nodal Explants

Category	LS means	Standard error	Lower bound (95%)	Upper bound (95%)	Groups
T0	57.222	0.386	56.457	57.987	D
T1	80.600	0.366	79.874	81.326	A
T2	78.300	0.366	77.574	79.026	B
T3	58.700	0.366	57.974	59.426	C
T4	40.800	0.366	40.074	41.526	G
T5	49.800	0.366	49.074	50.526	E
T6	44.300	0.366	43.574	45.026	F
T7	29.200	0.366	28.474	29.926	I
T8	31.400	0.366	30.674	32.126	H
T9	31.700	0.366	30.974	32.426	H
T10	28.200	0.366	27.474	28.926	I

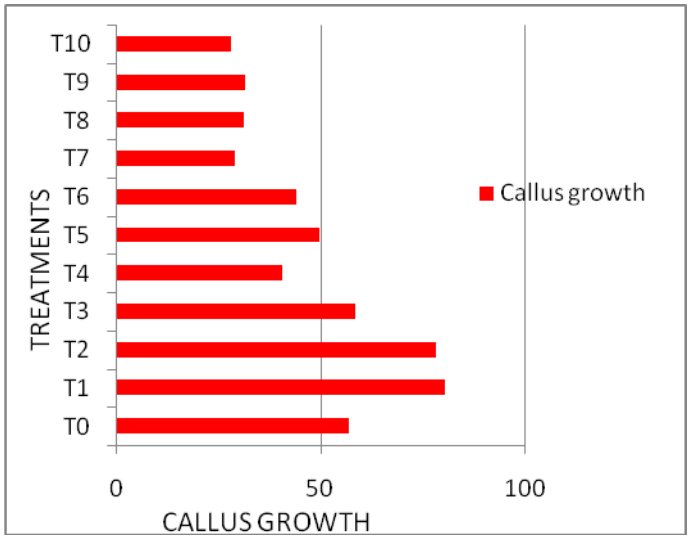

Chart - 2: Efficiency of Treatments in Nodal Explant

Table 8: Analysis of difference between treatments by Duncan's multiple range test – Apical shoot Explants

Category	LS means	Standard error	Lower bound (95%)	Upper bound (95%)	Groups
T0	57.556	0.477	56.610	58.502	D
T1	92.100	0.452	91.203	92.997	A
T2	80.600	0.452	79.703	81.497	B
T3	62.100	0.452	61.203	62.997	C
T4	50.600	0.452	49.703	51.497	E
T5	48.300	0.452	47.403	49.197	F
T6	41.100	0.452	40.203	41.997	G
T7	30.700	0.452	29.803	31.597	H
T8	31.000	0.452	30.103	31.897	H
T9	31.400	0.452	30.503	32.297	H
T10	25.700	0.452	24.803	26.597	I

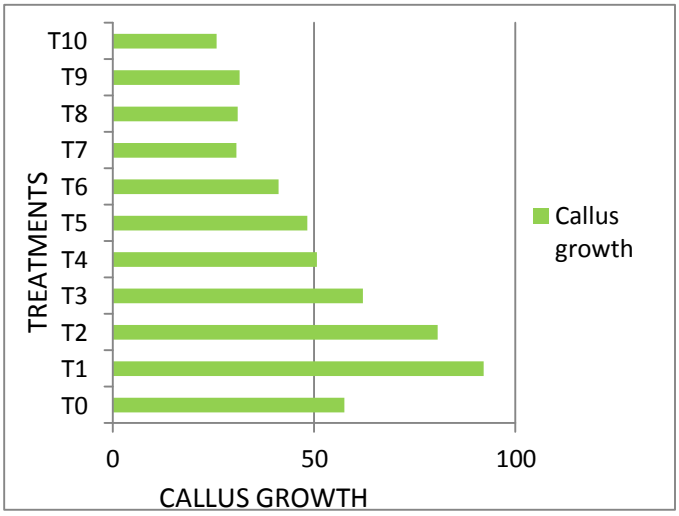

Chart - 3: Efficiency of Treatments in Apical Shoot Explant

Table 9: Analysis of difference between treatments by Duncan's multiple range test – Leaves Explants

Category	LS means	Standard error	Lower bound (95%)	Upper bound (95%)	Groups
T0	33.889	0.681	32.537	35.241	F
T1	70.700	0.646	69.417	71.983	A
T2	56.800	0.646	55.517	58.083	B
T3	48.900	0.646	47.617	50.183	C
T4	40.600	0.646	39.317	41.883	D
T5	38.300	0.646	37.017	39.583	E
T6	41.100	0.646	39.817	42.383	D
T7	29.700	0.646	28.417	30.983	G
T8	31.000	0.646	29.717	32.283	G
T9	30.300	0.646	29.017	31.583	G
T10	24.500	0.646	23.217	25.783	H

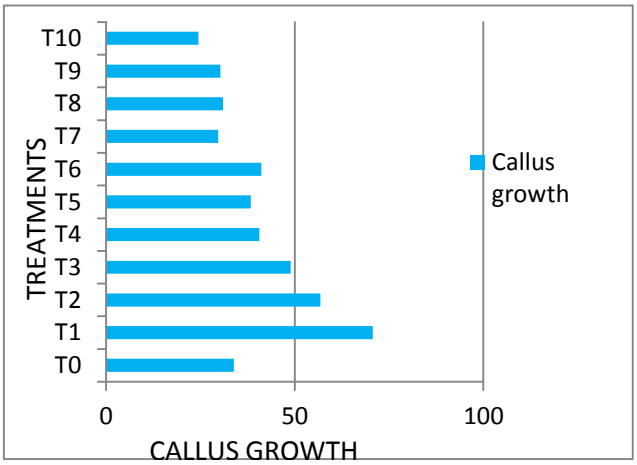

Chart - 4: Efficiency of Treatments in Leaves Explant

Table 10: Analysis of difference between treatments by Duncan's multiple range test – Petal Explants

Category	LS means	Standard error	Lower bound (95%)	Upper bound (95%)	Groups
T0	24.111	1.760	20.618	27.604	G H
T1	70.100	1.670	66.786	73.414	A
T2	35.200	1.670	31.886	38.514	D E
T3	32.000	1.670	28.686	35.314	E F
T4	40.600	1.670	37.286	43.914	C
T5	46.300	1.670	42.986	49.614	B
T6	39.200	1.670	35.886	42.514	C D
T7	28.700	1.670	25.386	32.014	F G
T8	27.300	1.670	23.986	30.614	F G
T9	24.000	1.670	20.686	27.314	G H
T10	21.900	1.670	18.586	25.214	H

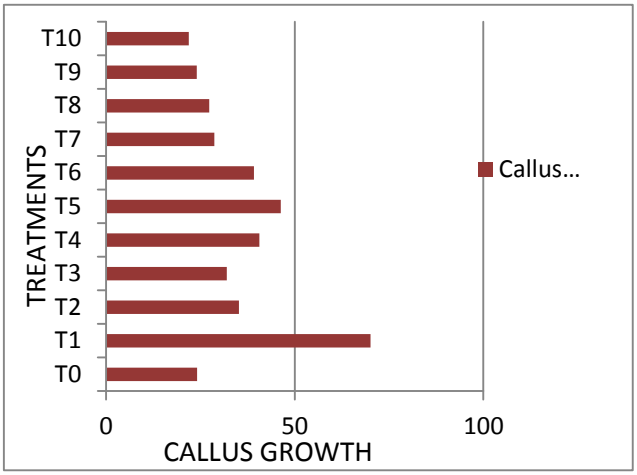

Chart - 5: Efficiency of Treatments in Petal Explant

The Duncan's Multiple Range test resulted in evaluating the LS means of higher percentage with T1 treatment for all explants. The performance was higher with Apical Shoot explant which resulted in 92% of callus growth other than all explants which had grown to 80%, 70% and 70% for Nodes, Leaves and Petals respectively. Further, the Polyphenol Exudation was continued with Apical Shoot.

a **b**

Fig - 1: Callus Growth
a. Pale Callus b. Healthy Callus

The figures represent the growth of callus in respective treatments which showed pale callus growth in all the treatments including control while the healthy green callus was healthy as a result of T1 treatment.

3.2 EFFECT OF POLYPHENOL EXUDATION:

3.2.1 Determination of callus growth after the elimination of polyphenols

Table - 11: Polyphenol exudation using silver nitrate – 1 - 5 mg/l:

EXPLANTS	POLYPHENOL EXUDATED				
	1 mg	2 mg	3 mg	4 mg	5 mg
Nodes	++++	+++	+++	+++	++
Leaves	++++	+++	+++	++	++
Petal	++++	+++	+++	+++	++
Apical shoot	+++	+++	++	++	-

Table - 12: Polyphenol exudation using PVP – 100 – 500 mg/l

EXPLANTS	POLYPHENOL EXUDATED				
	100 mg	200 mg	300 mg	400 mg	500 mg
Nodes	++++	+++	+++	++	+
Leaves	+++	+++	+++	+++	+++
Petal	+++	+++	+++	+++	++
Apical shoot	+++	+++	+++	+++	++

Table - 13: Polyphenol exudation using Activated charcoal - 100 – 500 mg/l

EXPLANTS	POLYPHENOL EXUDATED				
	100 mg	200 mg	300 mg	400 mg	500 mg
Nodes	++++	+++	+++	++	++
Leaves	+++	+++	+++	+++	++
Petal	+++	+++	+++	+++	+++
Apical shoot	+++	+++	++	++	+

Note: ++++ Very high; +++ High; ++ Moderately high ; + Low; - Nil

'++++' here represents that the callus induced very high polyphenol exudation; '+++' indicates that the callus induced high polyphenol exudation; '++' indicates the moderately high polyphenol exudation; '+' indicates low polyphenol exudation in callus; '-' indicates nil polyphenol exudation which was observed with Trial 5 that helped in the regeneration of callus into shoots. The other exudators were not effective for the elimination of polyphenols in 'Bhagwa' Pomegranate variety.

a b

Fig - 2: Polyphenol Exudation a. Before b. After

4. CONCLUSION

Apical shoots were the best part which has the ability to regenerate the callus into shoots. The initiation treatment for callus induction was proved in apical shoots explant in T1 treatment with green healthy callus when compared to the T2 and T3 treatments which produced the dull callus. The significance effects of the callus growth were calculated using one way ANOVA. Duncan's multiple range test proved the best treatment for the selected explants which showed 92% growth in apical Shoots; 80% growth in nodes, 70% growth in leaves and petals. The polyphenols exudation was eliminated by incorporating Silver nitrate of 5 mg/l in the MS + Sucrose (30g/l) + Adenine sulfate – 40mg/l + 6BAP – 5 mg/l. Indirect Organogenesis was achieved from the 'Bhagwa' pomegranate explants which have the ability to produce the disease free plants. 'Bhagwa' Pomegranate variety is very common among all other varieties which is recently known for Bacterial blight attack [15]. In order to bring back the Rich 'Bhagwa' pomegranate into the market. Micropropagation is the only effective way to produce disease free plants.

ACKNOWLEDGEMENTS:

My sincere thanks to my guide Dr. T. B. Sridharan, VIT University, Vellore who guided me technically in carrying out my Research work successfully.

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Publications

1. **T.B.Sridharan**, Shri Praksh, R.S. Chauhan, K.M. Rao, Kusum Singh and R. Naresh singh - Sensilla on the palps and legs of the adult ticks Argas Persicus Oken(Ixodoidea: argasidae and their projhections in to the central nervous system. *Int. J. Insect Morphol. And Embryol.* USA. **27(4)**: 273-289.1998
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13. Vickram, A. S., Ramesh Pathy, M., **Sridharan.T. B.** and J Srinivasan (2011). Optimization Of Semen Preparation Techniques Based On Rpm For Assisted Reproductive Technology *Journal of Pharmacy research* **4(8):** 2627-2632.
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31. Parameswari R, Ramesh Pathy M, Vickram A. S. and **T. B. Sridharan***, Smoking and Male Infertility: A Review