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RESPONSE OF DIFFERENT EXPLANTS OF WALNUT (*Juglans regia L.*) TO VARIOUS CONCENTRATIONS OF STERILANTS

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ABSTRACT

Walnut (Juglans regia L.)is considered to be one of the most recalcitrant species for invitro responses. To improve its micro propagation we introduce several modifications to current methods and evaluated the effect of different chemical sterilants. A complete protocol for sterilization of explants with maximum efficiency was developed to overcome the severe problem of contamination. The type, concentration and duration of exposure of a particular sterilant used were dependent on the plant material and the duration of treatment was chosen to minimize the tissue death. Sterilization of embryos was achieved by using 5% NaOCl for 15 minutes whereas surface sterilization of cotyledons was achieved on 5% NaOCl for 25 minutes and for other explants like shoot apices, leaves, apical and axillary buds 5% NaOCl for 25 minutes followed by 0.05% $HgCl_2$ for 5 minutes was found effective.

Keywords: Chemical Sterilants, Explants, Invitro, Sterilization, Walnut

I.INTRODUCTION

Walnuts are members of the relatively small *Juglandaceae* family, containing about 60 species, 21 of which are placed in the genus *Juglans*. Although native to Europe, it probably was not utilized there until improved forms were imported from Persia. Walnut grows well in areas with temperate climate and is produced commercially in 48 countries. In India, walnut is grown especially in North-Western Himalaya comprising states of Jammu & Kashmir, Himachal Pradesh and Uttaranchal between the elevations of 1,200 and 2,150m above MSL (Sharma and Dar, 2006)[1] but J&K is the principal walnut growing state having monopoly in the production of export quality nuts. The state has already created a special place in the international trade of walnut.

In forestry terms walnut is considered the noblest of the trees, because of its physical and chemical characteristics, together with its aesthetic qualities, which makes it a most beautiful and valuable tree. Throughout history, walnuts have been cherished as a health food and are a good source of proteins, vitamins, minerals, fatty acids, phytochemicals and walnut fibre and are a concentrated source of energy. These are very high in comparison to other foods as well as nuts. Walnuts in addition to these components also contain nine essential amino acids, which cannot be synthesised by the body at sufficient rate (Pandit, 2003)[2]. It is tree of great utility and almost every part of it is highly valued. Almost all plant parts of walnut are utilized in one way or the other but the fruit and timber have been put to maximum use by man.

Walnut generally does not respond favourably to the vegetative propagation methods under field conditions as is possible with majority of other temperate fruits. Walnut is a highly heterozygous plant and each seedling, therefore represents a different variety. In India Walnut cultivation is hampered by shortage of superior quality planting material, lack of standard propagation techniques, irregular orchards and pre and post-harvest management factors (Sharma and Dar, 2006)[1]. For a long time, propagation through seed was only method available for walnut multiplication, though this practice gives rise to highly variable plants.

Presently very little attention being paid in the valley for the production of these important fruit trees forces us to resort to some unconventional method of propagation and to see the potential of the technique for quicker propagation and uniform plantation of walnuts. The main application of walnut plant tissue culture could form a possible means of achieving more and faster propagation rate, genetic improvement and introduction of elite traits (special wood and nut quality) in the state. Hence, this study was undertaken with an aim to develop an approach for *in vitro* propagation of walnuts.

II.RESULTS

Mature walnuts (Seeds) were collected in the month of October from the pre identified selected trees. From those later on cotyledon fragments and embryonic axes were isolated in the lab for the experimental work. Other explants like shoot apices, leaf segments, nodal segments etc. were obtained either from field, controlled cultured plants or from green house collections. To avoid any bacterial and fungal growth which is detrimental to culture growth, explants were surface sterilized by chemical sterilants before transferring them on nutrient medium to establish *in vitro* axenic cultures. After using any of the surface sterilants for sterilization, plant materials were thoroughly washed several times with autoclaved water to ensure proper removal of sterilants.

2.1 Sterilization of Embryos

The effect of different sterilants used for standardizing the embryo sterilization technique of walnut is depicted in Table 1 and Fig. I. The most suitable chemical sterilant i.e. NaOCl (5%) for 15 minutes was only followed to get aseptic explants for culturing purpose throughout the study.

Table 1 Effect of different chemical sterilants on percent contamination and survival of walnut embryos[a] in an *in vitro* culture system.

Chemical sterilant	Concentration (% age)	Duration (min)	Contamination (%age)	Explant survival[b] (%age)
NaOCl	5.00	5	100	100
NaOCl	5.00	7	80	100
NaOCl	5.00	10	40	100
NaOCl	5.00	12	10	100
NaOCl	**5.00**	**15**	**0**	**100**
NaOCl	5.00	20	0	0
$HgCl_2$	0.01	5	100	100
$HgCl_2$	0.01	10	80	100
$HgCl_2$	0.05	5	100	60
$HgCl_2$	0.05	10	100	0
$HgCl_2$	0.10	10	60	60
$HgCl_2$	1.00	5	0	10

$HgCl_2$	1.00	10	0	10

a. Twenty replicates of each explant/treatment.

b. Culture contamination not taken into consideration.

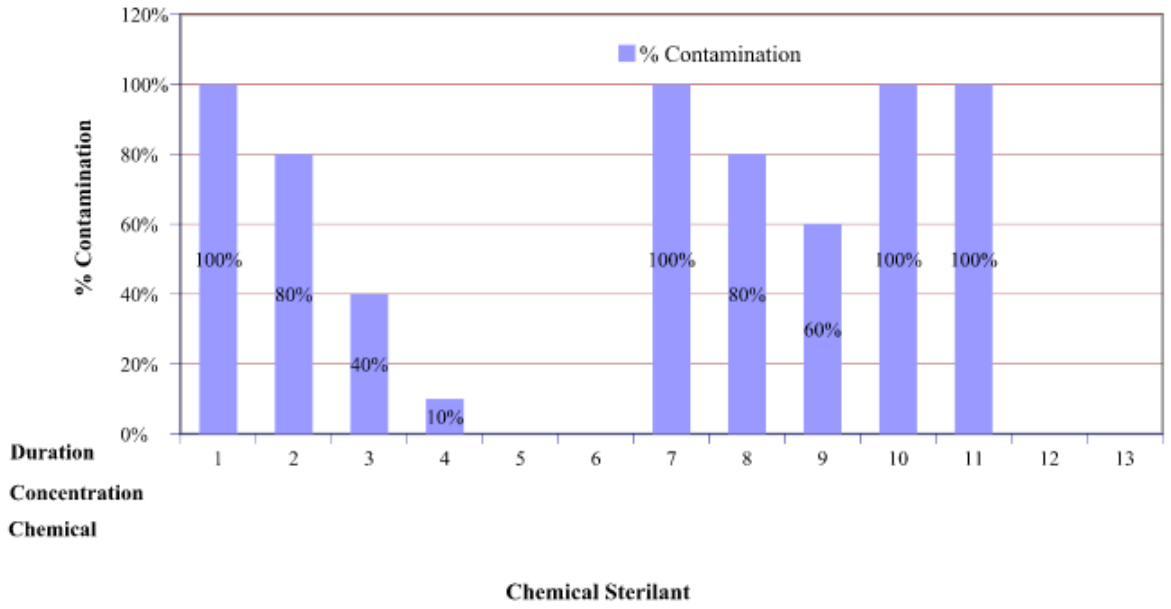

Fig. I Effect of different chemical sterilants on percent contamination and survival of walnut embryos*a* in an *in vitro* culture system.

2.2 Sterilization of Cotyledon

Cotyledons were excised from the nuts and testa was removed from the cotyledon pieces to reduce lethal browning. Chemical sterilants i.e. NaOCl and $HgCl_2$ were tried for different time periods at different concentrations as depicted in Table 2 and Fig. II. In this case sterilant NaOCl was found effective at 5% concentration when used for 25 minutes.

Table 2 Effect of different chemical sterilants on percent contamination and survival walnut cotyledons[a] in an *in vitro* culture system

Chemical sterilant	Concentration (%age)	Duration (min.)	Contamination (%age)	Explant[b] survival (%age)
NaOCl	1	30	100	80
NaOCl	2	10	100	80
NaOCl	2	20	100	80
NaOCl	5	10	100	100
NaOCl	5	15	80	80
NaOCl	5	20	20	80
NaOCl	**5**	**25**	**10**	**100**
NaOCl	5	35	0	40
$HgCl_2$	0.01	5	100	100
$HgCl_2$	0.01	10	100	100
$HgCl_2$	0.05	5	100	100
$HgCl_2$	0.05	10	90	70
$HgCl_2$	0.05	20	50	60

$HgCl_2$	0.10	5	80	60
$HgCl_2$	0.10	10	50	30

a. Twenty replicates of each explant/treatment.

b. Culture contamination not taken in to consideration.

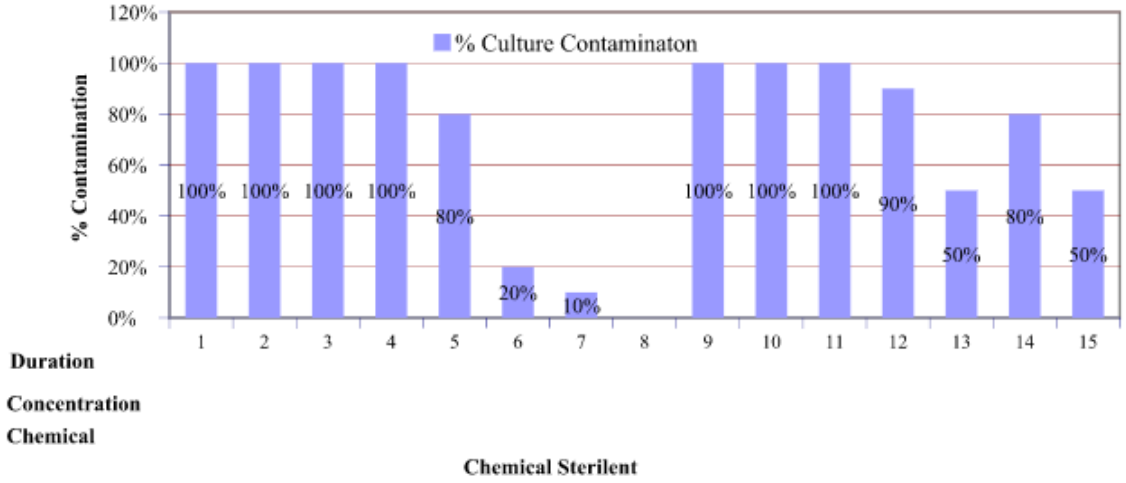

Fig. II Effect of different chemical sterilants on percent contamination and survival walnut cotyledons*a* in an *in vitro* culture system.

In vitro raised explants (like shoot tips, hypocotyl, roots, leaves) from *in vitro* raised seedlings needed no sterilization and were aseptically excised under laminar air flow cabinet and inoculated onto the nutrient medium.

2.3 Sterilization of Shoot Apices, Leaves, Axillary and Apical Buds

Explants like Shoot apices, Leaves, Axillary and Apical buds were surface sterilized with a range of sterilants and the concentrations of 5% NaOCl for 20 minutes followed by 0.05% $HgCl_2$ for 5 minutes proved effective for these explants as given in Table 3 and Fig. III. The sterilized explants were washed 3-4 times with autoclaved double distilled water to remove all the traces of sterilants. The exposed cut ends of explants about (0.5cm) were trimmed off to eliminate the toxic effect of sterilant which may have penetrated into the explants.

Table 3 Effect of different chemical sterilants on percent contamination and survival of different walnut explants (field material)[a] in an *in vitro* culture system.

Chemical sterilant	Concentration (% age)	Duration (min)	Contamination (% age)	Explant[b] survival (% age)
$HgCl_2$	0.01	10	100	100
$HgCl_2$	0.05	5	80	60
$HgCl_2$	0.05	10	40	20
$HgCl_2$	0.10	5	70	40
$HgCl_2$	0.10	7	60	20

$HgCl_2$	0.10	10	0	0
NaOCl	5	10	100	90
NaOCl	5	20	70	90
NaOCl	7	10	50	80
NaOCl	7	20	40	80
NaOCl	10	10	40	80
NaOCl	10	20	20	60
NaOCl $HgCl_2$	5 0.01	10 10	60	80
NaOCl $HgCl_2$	5 0.01	25 10	30	90
NaOCl $HgCl_2$	5 0.01	25 7	20	60
NaOCl $HgCl_2$	**5** **0.05**	**20** **5**	**10**	**80**
NaOCl $HgCl_2$	5 0.05	25 5	0	50

a. Twenty replicates of each explant/treatment.

b. Culture contamination not taken into consideration.

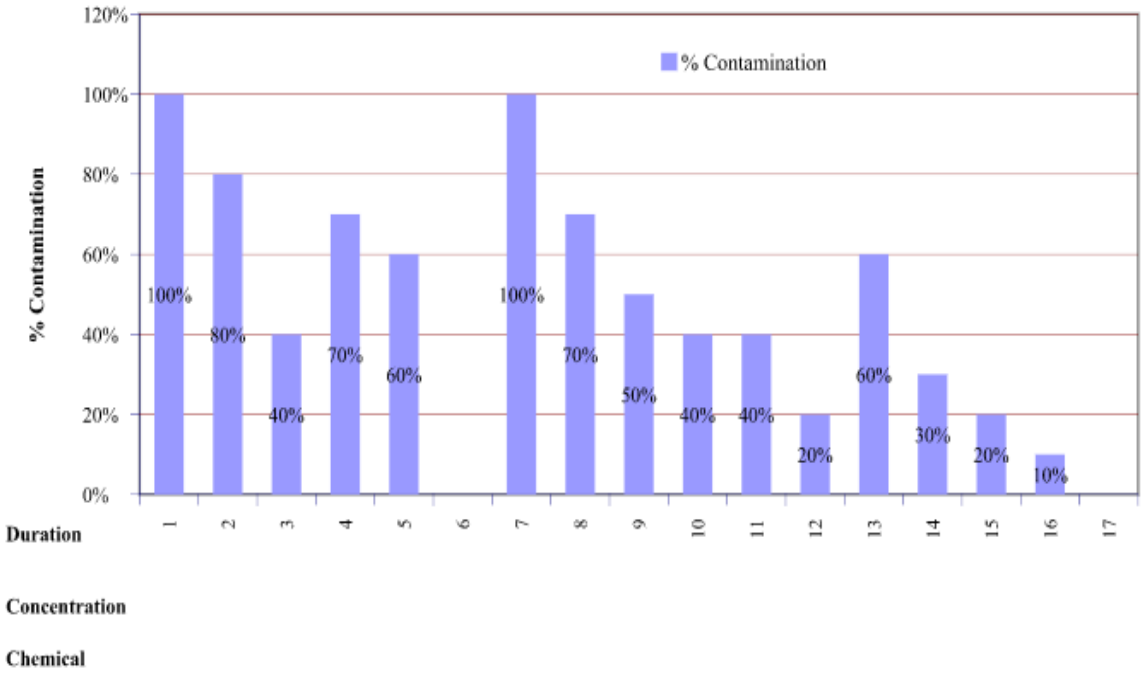

Fig. III Effect of different chemical sterilants on percent contamination and survival of different walnut explants (field material)*a* in an *in vitro* culture system.

III.CONCLUSION

The establishment of initial cultures has always been a difficult task. To overcome the grave problem of microbial infection aseptic technique is important for success of plant cell, tissue and organ culture. The growth of micro-organisms on the culture media is fast due to presence of sucrose in the media which ultimately leads to death of explant. To prevent microbial growth, sterilization of explants is important. A complete protocol for sterilization of explants with maximum efficiency was developed to overcome the severe problem of contamination. Plant parts or seeds exposed outside carry contaminated substances. *Ex vitro* raised explants express high degree of fungal and bacterial contamination. In such cases to prevent microbial growth in culture media, sterilization is must and while perusing the literature, it was noticed that different workers like *Heile-Sudholt* (1986)[3], Penula *et al.* (1987)[4], Neuman *et al.* (1993)[5] and Long *et al.* (1995)[6] tried different surface sterilants for achieving sterilization of different explants of *Juglans regia.* However, in present study different concentrations of NaOCl and $HgCl_2$ for different time durations were tried for sterilization of walnut (*Juglans regia)* explants and the maximum sterilization of embryos was achieved by using NaOCl for 15 minutes and for other explants like shoot apices, nodal segments, leaves, axillary and apical buds 5% NaOCl for 20 min followed by 0.05% of $HgCl_2$ for 5 min proved effective and for cotyledon sterilization NaOCl was found effective at 5% concentration for 25 min.

IV. Acknowledgement

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