INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH

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STUDIES ON THE EFFECT OF SURFACE STERILIZATION TIME AND PLANT BIOREGULATORS ON *IN VITRO* REGENERATION OF BULB SCALE EXPLANTS OF HYBRID LILIUM CV. SORBONNE

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ABSTRACT
The present experiment was conducted to find out the most suitable surface sterilizing timing of 0.1% $HgCl_2$ for controlling contamination in bulb-scale explants of hybrid Lilium Cv. Sorbonne using MS media as base with hormones BAP (1.0 mg/l), 2, 4-D (0.5 mg/l). Among the various sterilization timing, the bulb scale explants sterilized with 0.1% $HgCl_2$ for 6 min significantly reduced the fungal contamination (6.67%), bacterial (6.66%), death (6.67%).The explants treated with 0.1% $HgCl_2$ for 6 min significantly enhanced the aseptic culture by 86.67% and survival (80.00%). For callus induction, media was supplemented with BAP (0.5 mg/l, 1.0 mg/l) and 2, 4-D (0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l) alone and in combinations, along with a control. The results after 30 days revealed that MS medium fortified with 1.5 mg/l, 2,4-D significantly advanced the days for callus induction acquiring 14.33 days for callusing with the maximum percentage of callus production (80.00%).The treatment also increased the callus spread (0.26 cm × 0.16 cm). After subculture, MS medium supplemented with 1.5mg/l, 2, 4-D increased the callus spread (0.3 cm×0.26 cm) and produced compact light green calli mass. Hence 6 min was the best for surface sterilization timing with 0.1% $HgCl_2$ and MS medium supplemented with 2,4-D,1.5 mg/l resulted in maximum callus formation. *In vitro* propagation of this highly lucrative cut flower is of great importance for the mass supply of disease-free planting materials.

KEYWORDS: Bacterial, Bulb, Fungal, $HgCl_2$, Sterilization.

INTRODUCTION

Lilium is one of the leading cut flower crops in the world (Dole and Wilkins, 1999). It ranks 4th in the international flower trade (Anonymous, 1996). Being derived from inter specific hybridization its importance as a bulbous crop in the commercial market has increased a lot. Lilium hybrids are famous for colour, size, and fragrance. These have straight stems for a height of about 30-36" tall, high bud counts and generally brightly spotted blossoms with a variable in shape from simple open bowls to flowers with re-curved petals. Colours of hybrid Lilium range from the softest pastels to fiery reds and pinks which practically glow as the sun shines on them. Lilium tissues in general, have a high regeneration potential. Bulb and scales are most commonly used as explants for vegetative propagation. Unfortunately, being under-ground parts, there is a high contamination risk with bulb scales and it only rise to a single small plant forming a single bulb only. Through tissue culture, there is not only a continuous supply of bulblets but true to type and disease free plantlets can be obtained (Pandey *et al.,* 2009). Keeping in view the importance and high demand for these lucrative cut flowers cultivar the present, experiment was carried out to find out the best timing for surface sterilization with 0.01% $HgCl_2$ and to standardize the plant bioregulators for callusing and further production of plantlets.

MATERIALS & METHODS

The investigation was carried out at the Biotechnology-cum- Tissue Culture Center, OUAT, Bhubaneswar. The chemicals used for the present study were analytical reagents of excel R grade of Merck (India), Qualigen fine Chemicals, and Himedia Laboratories Ltd. (India). Auxins, Cytokinins, Myo-inositol and Fe-EDTA were from Sigma (USA) and Agar from Himedia Lab Ltd (India). For the preparation of MS culture medium (Murashige and Skoog, 1962) required quantities of macronutrients, micronutrients, Fe-EDTA, vitamins and plant bio regulators were taken from the stock solution and required quantity of sucrose dissolved in water was added fresh to the medium. The pH of the solution was adjusted to 5.7 ±0.1 using 0.1N NaOH or 0.1 N HCl. Then volume was made up to 1 liter with distilled water. Agar (0.6% w/v) was added to the medium boiled and poured to the culture tubes and plugged with non-absorbent cotton. Plugged culture tubes containing culture medium were autoclaved for the 20 minutes at 121^{0}C and 15 Psi pressure. The autoclaved medium was kept in a laminar air flow bench for cooling. All the glassware were dipped in the detergent solution for overnight and washed under running tap water. They were rinsed with distilled water and then dried in an oven for 2hrs at 150^{0}C. Forceps, petridishes and scaples were thoroughly cleaned with isopropanol or rapped with paper and kept in a clean sterilized in the autoclave at 15 psi and 121^{0}C for 20

minutes. The working chamber of laminar air flow cabinet was wiped with isopropanol. Filtered air (80-100 cft/min) to ensure that particles do not settle in working area was blown for 5min. The sterilized materials to be used (except living tissue) were kept made the chamber and exposed to UV light for 30 minutes. While working, filtered air was continuously passed the laminar air flow cabinet. Pre-cooled bulbs (20^0C for 8 weeks) of hybrid Lilium Cv. Sorbonne was collected from the mother plant maintained at the green house of the Biotechnology-cum- Tissue Culture Center, OUAT, Bhubaneswar. The roots which emerged on the rhizomes were trimmed with the scalpel blade. The bulbs scales were then excised from the bulbs and the lower portion of the inner most scales were used as explants. The bulbs were washed thoroughly in running tap water for 30 minutes, followed by twenty 20 for 15 min. Further aseptic surface sterilization was carried out with 2% bavistin with constant stirring which was then rinsed out after 15 min with sterile distilled water for three times and these were divided into 9 groups. These were then treated with surface sterilant tween 20 for 20 min. and then rinsed with distilled water 3 times with sterile airflow in the laminar hood. The sterilized explants were then prepared by removing the rhizome and separating the scales into individuals, subjected to further 0.1% $HgCl_2$ solution treatment for different timing (3 min, 4 min, 5 min, 6 min, 7 min, 8 min and 9 min) and a control (without $HgCl_2$ treatment). These sterilized explants were then cultured on Murashige and Skoog (1962) medium supplemented with plant bioregulators BAP (1.0 mg/1) and NAA (0.5mg/1) with 8% (w/v) agar, 30% (w/v) sucrose replicated thrice. The observation on the following parameters was recorded after 15 days after inoculation (DAI), 30 (DAI) and 45 (DAI) on Fungal %, Bacterial % contamination, death %, survival % and aseptic %.

For callus formation study, explants were treated with the best surface sterilizing time among the surface sterilization treatments taken into consideration. After the disinfection each section of the bulb scale (7 × 7 mm), with the dorsal side in contact with the medium, was placed in a culture tube with MS (Murashige and Skoog,1962) medium supplemented with BAP at 0.5 mg/l or 1.0 mg/l, in combinations of 2,4-D at 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l or 2.5mg/l and alone of 2,4-D of same concentrations, were also taken excluding BAP. Observation on days to callus initiation, % of callus produced, callus spread were recorded from 30 DAI and callus spread, colour of the callus and nature of callus recorded at 60 DAI. After inoculation, the cultures were kept, at 25 ±20°C in an air conditioned room with a 16 hours photo period (3000-3200 lux) light intensity and 80% relatively humidity.

All the trails of the experiments were replicated thrice and 10 culture tubes per replication in each treatment were taken. The raw data obtained during the experimental observations were subjected to completely randomized design (Gomez and Gomez, 1984). The significance and non- significance of the treatment effect were judged with the help of 'F' variance ratio test. Calculated 'F' value was compared with the table value of 'F' at 5% level of significance. The data were transferred from where ever required before suitability of Analysis of Variance (ANOVA) analyzed in statistical package SAS version 7.0.

RESULTS & DISCUSSION

Explant disinfection

Sterilization time response of the bulb scale explants of hybrid Lilium cv. Sorbonne with 0.1% $HgCl_2$with different timing depicted in table 1 revealed that among the different timing for the bulb scale explants after 15 days after inoculation (DAI), fungal contamination were recorded maximum at T_1 (tap water) (30.00%) followed by T_2 (3 min) (23.33%) where as the minimum contamination was recorded at T_5 (6 min), T_6 (7 min), T_7 (8 min) and T_8 (9 min) (6.67%).

TABLE 1: Effect of Surface sterilization time on the level of contamination, aseptic culture and survival of bulb scale explants of hybrid Lilium Cv. Sorbonne after 15 DAI (Days after inoculation)

Sl. No.	Treatments	Fungal %	Bacteria %	Death %	Aseptic %	Survival %
1	$T_{1\ (Tap\ Water)}$	30.00 (33.21)	10.00 (18.44)	0.00 (2.50)	63.33 (52.89)	60.00 (50.77)
2	$T_{2\ (3\ min)}$	23.33 (29.07)	10.00 (18.44)	3.33 (10.95)	66.67 (55.12)	63.33 (52.89)
3	$T_{3\ (4\ min)}$	16.67 (24.53)	13.33 (21.63)	3.33 (10.95)	70.00 (56.79)	66.67 (55.12)
4	$T_{4\ (5\ min)}$	16.67 (24.53)	3.33 (10.95)	3.33 (10.95)	80.00 (63.44)	76.67 (61.56)
5	$T_{5\ (6\ min)}$	6.67 (15.66)	0.00 (2.5)	3.33 (10.95)	93.33 (75.33)	90.00 (71.56)
6	$T_{6\ (7\ min)}$	6.67 (15.66)	3.33 (10.95)	13.33 (26.63)	90.00 (71.56)	76.67 (61.56)
7	$T_{7(8\ min)}$	6.67 (15.66)	3.33 (10.95)	23.33 (29.07)	90.00 (71.56)	66.67 (55.12)
8	$T_{8\ (9\ min)}$	6.67 (15.66)	3.33 (10.95)	23.33 (29.07)	90.00 (71.56)	66.67 (55.12)
	SEm (±)	4.16	-	2.79	2.11	2.98
	CD at 5%	11.53	-	7.72	5.84	8.26

**Figures in parenthesis* are *arc sin transformed values.*

The bacterial contamination was recorded maximum at T_3 (4 min) (13.34%) and the minimum were recorded at T_5 (6 min) (0.00%). Death recorded maximum at T_7 (8 min), T_8 (9 min) (23.33%) and the minimum was recorded at T_2 (3 min), T_3 (4 min), T_4 (5 min), T_5 (6 min) with a reading of (3.33%). Maximum aseptic cultures were recorded at T_5 (6 min) (93.33%) and minimum was observed in the T_1 (tap water) (63.33%). Survival percentage of explants were noted maximum at T_5 (6 min) (90.00%) and minimum were obtained at T_1 (tap water) (60.00%). After 30 days after inoculation in table.2 it was revealed that the maximum fungal contamination was recorded at T_1 (tap

water) (30.00%) and the minimum at T_5 (6 min), T_6 (7 min), T_7 (8 min), T_8 (9 min) with (6.67%). Bacterial contamination was maximum at T_1 (tap water), T_2 (3 min), T_3 (4 min) (13.33 %) and the minimum at T_5 (6 min) with (3.33%). Maximum death of explants was recorded at T_7 (8min), T_8 (9 min) (23.33%) and the minimum were observed at T_1 (tap water) (0.00%). In case of aseptic culture, maximum was recorded at T_5 (6 min), T_6 (7 min) and T_7 (8 min) with (90.00%) and minimum was recorded at T_1 (tap water) (60.00%). Survival was recorded maximum at T_5 (6 min) (86.67%) and minimum survival was recorded at T_1 (tap water) (56.67%). After 45 days of inoculation from table 3 revealed that the fungal contamination was maximum at T_1 (tap water) (30.00%) and the minimum was at T_5 (6 min), T_6 (7 min), T_7 (8 min) and T_8 (9 min) with (6.67%). Bacterial contamination was maximum recorded at T_1 (tap water) (20.00%) and with a minimum of (3.33%) in T_6 (7 min) and T_8 (9 min). Maximum death was recorded at T_7 (8 min) and T_8 (9 min) (26.67%) with a minimum of death in T_1 (tap water) (0.00%) followed by, T_2 (3 min), T_3 (4 min) and T_4 (5 min) with (3.33%). In case of aseptic culture, maximum was recorded at T_6 (7 min) and T_7 (8 min) (90.00%) and the minimum was recorded at T_1 (tap water) (50.00%). The maximum explants survived at T_5 (6 min) (80.00%) followed by T_6 (7min) (73.33%) and the minimum survival was recorded at T_1 (Tap water) (50.00%). The results show that treatment T_5 (6 min) reduced the contamination and resulted in minimum phytotoxicity to the Lilium bulb scale explants and achieve maximum infection free culture (Rihan *et al*., 2002).The results are in alignment with the finding of Beura *et al*. (2016) in *Bahunia galpinii*.

TABLE 2: Effect of Surface sterilization time on the level of contamination, aseptic culture and survival of bulb scale explants of hybrid Lilium Cv. Sorbonne after 30 DAI

Sl. No.	Treatments	Fungal %	Bacteria %	Death %	Aseptic %	Survival %
1	$T_{1\ (Tap\ Water)}$	30.00 (33.21)	13.33 (26.63)	0.00 (2.50)	60.00 (50.77)	56.67 (49.21)
2	$T_{2\ (3\ min)}$	23.33 (29.07)	13.33 (26.63)	3.33 (10.95)	63.33 (52.89)	60.00 (50.77)
3	$T_{3\ (4\ min)}$	16.67 (24.53)	13.33 (26.63)	3.33 (10.95)	70.00 (56.79)	66.67 (55.12)
4	$T_{4\ (5\ min)}$	16.67 (24.53)	10.00 (18.44)	3.33 (10.95)	73.33 (59.07)	70.00 (56.79)
5	$T_{5\ (6\ min)}$	6.67 (15.66)	3.33 (10.95)	3.33 (10.95)	90.00 (71.56)	86.67 (69.09)
6	$T_{6\ (7\ min)}$	6.67 (15.66)	3.33 (10.95)	13.33 (26.63)	90.00 (71.56)	76.67 (69.09)
7	$T_{7(8\ min)}$	6.67 (15.66)	3.33 (10.95)	23.33 (29.07)	90.00 (71.56)	66.67 (55.12)
8	$T_{8\ (9\ min)}$	6.67 (15.66)	6.67 (15.66)	23.33 (29.07)	86.66 (69.01)	63.33 (52.89)
	SEm (±)	3.81	-	3.11	4.56	2.99
	CD at 5%	10.57	-	8.64	12.62	8.26

**Figures in parenthesis* are *arc sin transformed values.*

TABLE 3: Effect of Surface sterilization time on the level of contamination, aseptic culture and survival of bulb scale explants of hybrid Lilium Cv. Sorbonne after 45 DAI

Sl. No.	Treatments	Fungal %	Bacteria %	Death %	Aseptic %	Survival %
1	$T_{1\ (Tap\ Water)}$	30.00 (33.21)	20.00 (26.56)	0.00 (2.50)	50.00 (45.00)	50.00 (45.00)
2	$T_{2\ (3\ min)}$	23.33 (29.07)	16.67 (24.53)	3.33(10.9 5)	60.00 (50.77)	56.67 (49.21)
3	$T_{3\ (4\ min)}$	16.67 (24.53)	13.33 (26.63)	3.33 (10.95)	70.00 (56.79)	66.67 (55.12)
4	$T_{4\ (5\ min)}$	16.67 (24.53)	13.33 (26.63)	3.33 (10.95)	70.00 (56.79)	66.67 (55.12)
5	$T_{5\ (6\ min)}$	6.67 (15.66)	6.66 (15.55)	6.67 (15.66)	86.67 (69.09)	80.00 (63.44)
6	$T_{6\ (7\ min)}$	6.67 (15.66)	3.33 (10.95)	16.67 (24.53)	90.00 (71.56)	73.33 (59.07)
7	$T_{7(8\ min)}$	6.67 (15.66)	3.33 (10.95)	26.67 (31.47)	90.00 (71.56)	63.33 (52.89)
8	$T_{8\ (9\ min)}$	6.67 (15.66)	6.67 (15.66)	26.67 (31.47)	86.67 (69.09)	60.00 (50.77)
	SEm (±)	4.31	2.33	3.50	2.98	2.72
	CD at 5%	11.96	6.48	9.69	8.26	7.54

**Figures in parenthesis* are *arc sin transformed values*

Callus Initiation

The data presented in the table.4, recorded that explants cultured at T_5 and T_6 significantly reduced the days to callusing (14.00) followed by T_4 (14.33) day, maximum callus percentage was recorded at T4 (80.00), followed by T_3, (76.60). The callus spread was maximum (0.26×0.16 cm) in T4.

In subculture 1, the data presented in table 4. revealed that MS medium fortified with 2, 4-D 1.5mg/l alone significantly proliferated the callus spread (0.5×0.4 cm), followed by T_4 (0.3×0.26 cm), similar trend was also reported for the colour of callus and nature of callus MS medium supplemented with 2,4-D 2.5 mg/l and 1.5 mg/l alone gave light green compact callus with good spread.

The result obtained in the investigation for the callus initiation is supported by the findings of Gochhayat *et al*., (2017) who worked on the callusing of hybrid Lilium cv Fangio. The difference obtained in the requirements of plant bioregulators as reported by Beura *et al*. (2016), may be due to the difference in endogenous phytohormones and interaction between various factors or supplied in the medium (Hartmann and Kaster, 1983).

TABLE 4: Effect of Plant bioregulators on callus emergence, development, nature of callus, callus spread and callus colour of bulb scale explants of hybrid Lilium Cv. Sorbonne

Characters →				Culture-1 (30 days)			Subculture-I	(60 Days)
Treatments ↓	BAP	2,4-D	Days to Callus initiation	% of Callusing	Size of the Callus (cm)	Size of Callus (cm)	Colour of Callus	Nature of Callus
T_1	-	-	14.33	60.00(50.77)	0.10×0.10	0.20×0.16	Light green	Compact
T_2	-	0.5	15.00	73.33(59.07)	0.10×0.20	0.20×0.23	Light green	Compact
T_3	-	1.0	15.66	76.66(61.49)	0.20×0.13	0.20×0.23	Light green	Compact
T_4	-	1.5	14.33	80.00(63.44)	0.26×0.16	0.50×0.40	Light green	Compact
T_5	-	2.0	14.00	73.33(59.07)	0.04×0.26	0.03×0.26	Light green	Compact
T_6	-	2.5	14.00	70.00(56.79)	0.03×0.16	0.43×0.33	Light green	Compact
T_7	0.5	0.5	21.00	50.00(45.00)	0.10×0.16	0.20×0.23	White	Compact
T_8	0.5	1.0	21.33	46.66(43.41)	0.20×0.16	0.20×0.26	White	Compact
T_9	0.5	1.5	22.00	46.66(43.41)	0.20×0.23	0.30×0.33	White	Compact
T_{10}	0.5	2.0	23.00	53.33(47.07)	0.20×0.26	0.30×0.30	White	Compact
T_{11}	0.5	2.5	23.33	60.00(50.77)	0.20×0.16	0.30×0.30	White	Compact
T_{12}	1.0	0.5	23.00	53.33(47.07)	0.10×0.10	0.20×0.20	White	Compact
T_{13}	1.0	1.0	23.00	56.66(49.15)	0.10×0.16	0.20×0.23	White	Compact
T_{14}	1.0	1.5	24.33	56.66(49.15)	0.16×0.10	0.30×0.20	White	Compact
T_{15}	1.0	2.0	24.66	60.00(50.77)	0.26×0.16	0.33×0.26	White	Compact
T_{16}	1.0	2.5	24.00	56.66(49.15)	0.23×0.13	0.30×0.26	White	Compact
SEm (±)			0.22	2.64				
CD 5%			0.62	7.45				

Figures in parenthesis are *arc sin transformed values*

A **B** **C** **D**

Plate A and B- Mother plant of Hybrid Lilium Cv. Sorbonne, **Plate C and D**- Bulb and bulb scale explant of Hybrid Lilium Cv. Sorbonne

Plate E- Callus intiation from the bulb scale explant of Hybrid Lilium Cv. Sorbonne.

Plate F- Shoot initiation from the callus of bulb scale explant of Hybrid Lilium Cv. Sorbonne

CONCLUSION

It was concluded that for *in vitro* propagation of hybrid Lilium Cv. Sorbonne bulbs scale explants sterilization with $HgCl_2$ at 0.1% for 6 min is more effective for getting maximum aseptic culture along with the survival of bulb explants. Callus growth from the explants in the MS medium supplemented with 2,4-D at 1.5 mg/l and 2.0 mg/l alone was found to be the most effective combinations plant bioregulator for the purpose of mass multiplication with in short period of time.

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