Original Research Article **Pathology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

STANDARDIZATION OF STERILIZATION TIME AND PLANT BIOREGULATORS FOR CALLUS FORMATION IN HYBRID LILIUM CV. FANGIO

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ABSTRACT

An efficient protocol was developed for the bulb scale explants derived callus culture for hybrid Lilium Cv. Fangio under *in vitro* conditions on Murashige and Skoog basal medium in the Biotechnology-cum-Tissue Culture Centre, Orissa University of Agriculture and Technology, Bhubaneswar. The bulb scale explants were treated with 0.1 % $HgCl_2$ (3 min, 4min, 5min, 6 min, 7min, 8min and 9 min) and control (without treatment) and cultured on MS media, among the treatments, 5 minutes timing resulted in minimum contamination [fungal % (6.67), bacterial % (6.67)] maximum survival % (83.33%). The best surface sterilization time was further taken into consideration for treatment of explants treated for sterilization and cultured in the Basal media supplemented with BAP (0.5, 1.0 mg/l) in combination with 2,4-D (0.5,1.0,1.5,2.0, and 2.5 mg/l) and 2,4-D (0.5,1.0,1.5,2.0, and 2.5 mg/l) alone with control. Basal media supplemented with BAP (1.0 mg/l) and 2,4-D (2.0 mg/l) produced maximum callus % and spread, profuse green callus was also recorded in similar combination which opened prospects for developing an indirect means of *in vitro* regeneration of hybrid Lilium Cv. Fangio there by strengthening the way biotechnology which could be used for improvement and satiate the national and international demands of this cut flowers.

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

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Received on: 14.12.2017
Revised and Accepted on: 30-03-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.2.b613-617

INTRODUCTION

Lilium being a monocot from Liliaceae family is one of the most important bulbous crops. Apparently, 80 species of Lilium are found in the temperate and subtropical zones of northern hemisphere[1]. It is one of the leading cut flower crops to be known in the world for its beauty, a fascinating form of flowers, long vase life and capacity to rehydrate after long transportation. Fangio belongs to the Lilium hybrid varieties is a perennial plant and do have a miraculous appeal for its colour that goes from deep purple to red with a height of 120-130 cm in 80-90 days with very strong supporting stem and up-facing flower with a long vase life. Flower bulbs have been appreciated and cultivated for thousands of years and long before they were widely grown commercially or extensively researched. Previous literature suggests that explants from flower organs to bulb scales of Lilium could be easily manipulated and regenerated using tissue culture techniques[2]. Lilium propagation is usually done by vegetative means which produces 3-4 bulbs per bulb scale depending on size and variety. The multiplication efficacy by bulb is low and the plantlets are more susceptible to diseases. Therefore, there is a need to develop a protocol for its mass propagation. Through tissue culture, there is not only a continuous supply of bulblets but true-to-type and disease free plants can be obtained. Therefore the objective of the present research was to establish a protocol for standardization of sterilization time and Plant bioregulators for callus formation in Lilium hybrid Cv. Fangio.

MATERIALS AND METHODS

The present investigation was carried out during the year 2013-14 at Biotechnology-cum-Commercial Tissue Culture Centre, OUAT, Bhubaneswar.

Source of Explants

For this study, Healthy and disease free bulbs of hybrid Lilium Cv. Fangio was collected from fresh plants grown under Polyhouse in the of Biotechnology-cum-Commercial Tissue Culture Center, OUAT, Bhubaneswar, maintained as Mother plant for conducting *in vitro* culture work in the laboratory. The bulb scale was taken for the *in vitro* culture.

Stock solution, Media preparation and Sterilization

The chemicals used for the present study were analytical reagents of excel R grade of Titan Biotech Ltd., Ranbaxy Laboratory Ltd., Merck (India), Qualigen Fine Chemicals, and Himedia Laboratories Ltd. (India). Auxins, Cytokinins, myo-inositol and Fe-EDTA were supplied by Sigma (USA) and Agar from Ranbaxy Laboratory Limited. MS Medium (Murashige and Skoog, 1962)[3] was used throughout the investigation, required quantities of macronutrients, micronutrients, Fe-EDTA, vitamins and plant bioregulators were taken from the stock solution and required quantity of sucrose dissolved in distilled water was added fresh to the medium. The pH of the solution was adjusted to 5.7$\pm$ 0.1 using 0.1N NaOH or 0.1 N HCL. Then volume was made up to 1 L with distilled water. Agar (0.6% w/v) was added to the medium boiled and poured into the culture tube and plugged. Culture tube containing culture medium was autoclaved for the 20 minutes at 121^{0}C and 15 Psi pressure. The autoclaved medium was kept in 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, Petridis and scalpel were thoroughly cleaned with isopropanol or rapped with paper and kept in a clean sterilized in an 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 5 minutes. The sterilized materials to be used (except living tissue) were kept in the chamber and exposed to UV light for 30 minutes.

Inoculation

The sterilized explants were then finally prepared by removing the rhizome and separating the scales into individuals. Continuing with the above conditions these explants were then subjected to further to 30 min tap water washing and followed by 15 minute tween 20 solution washing, further the explants was treated with 2 % bavistin for 20 min and as per the treatment plan the explants was treated with 0.1% $HgCl_2$ (3 min, 4min, 5min, 6 min, 7min, 8min and 9 min) and a control (without treatment). These sterilized explants were then cultured on Murashige and Skoog (1962)[3] medium supplemented with growth hormones BAP (1.0 mg/l) and NAA (0.5mg/l) with 8% (w/v) agar, 30% (w/v) sucrose with three replications. The data recorded for different parameters i.e. fungal %, bacterial %, death %, aseptic % and survival % of explants were recorded from 15 DAI (Days after inoculation), 30 DAI and 45 DAI. Later the treatment having the highest survival percentage with minimum infection and death percentage were taken as the standard time for disinfecting explants before inoculating them for *in vitro* propagation. For callus formation study explants were treated with the best surface time (5 minute) among the surface sterilization treatments were 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)[3] medium containing BAP (0.5, 1.0 mg/l) in combination with 2,4-D (0.5,1.0,1.5,2.0, and 2.5 mg/l) and 2,4-D (0.5,1.0,1.5,2.0, and 2.5 mg/l) alone and a control. 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.

Establishment of culture

After inoculation, the culture was kept at 25$\pm$2^0 C in an air conditioned room with a 16 hours light period (3000-3200 lux) supplied by fluorescent tubes and 80% relatively humidity[4].

STATISTICAL ANALYSIS

The raw data obtained during the experimental observations were subjected to statistical analysis as per method by Gomez and Gomez, (1984)[5]. The

significance and not- 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 AND DISCUSSION

Sterilization time response of bulb scale explants of Hybrid Lilium Cv. Fangio in response to the time of disinfection is depicted in table.1 to 3. Data revealed that among the different timing for the tissue cultured bulb scale explants at 15 DAI (Days after inoculation), the maximum fungal contamination % recorded at T_1 (16.67%) in control (Tap-water) and T_2 (3 min), minimum fungal contamination % recorded at T_4 (5 min), T_5 (6 min), T_6 (7 min), T_7 (8 min) and T_8 (9 min) with (3.33%). Bacterial contamination was recorded maximum at T_1 (tap water) (6.66%) was minimum was recorded at T_4 (5 min), T_5 (6 min) and T_6 (7 min) with (0.00%). In case of explants death, maximum was recorded at T_8 (9 min) (23.34%) and the minimum was recorded at T_2 (3 min), T_3 (4 min) and T_4 (5 min) (3.33%). In case of aseptic culture, maximum was recorded at T_4 (5 min), T_5 (6 min) and T_6 (7 min) (96.67%) and the minimum were obtained in T_1 (76.67%). Maximum survival percentage of explants were obtained at T_4 (6min) (93.33%) and minimum at T_1 (66.67%). After 30 DAI (Table.2) it was observed that the fungal contamination % was maximum at T_1 (20.00%) and minimum at T_4 (5 min), T_5 (6 min), T_6 (7 min), T_7 (8 min), T_8 (9 min) (3.33%). In case of bacterial contamination % maximum was at T_3 (4 min) (16.67%) and the minimum at T_6 (7min) (3.33%). In Case of Death % of explants was recorded maximum at T_8 (30.00%) and the minimum at T_2 (3 min) (3.33%), similar results were also obtained at T_4 and T_5. In case of aseptic culture maximum was recorded at T_6 (7 min) (93.34%) and the minimum was recorded at T_1 (tap water) (66.67%). Survival % was maximum at T_4 (5 min) (86.67%) and minimum was recorded at T_1 (56.67%). At 45 DAI (Table.3) fungal contamination % was maximum recorded at T_1 (tap water) and T_2 (3 min) with (20.00%) and minimum was recorded at T_5 (6 min), T_6(7 min),T_7 (8 min) and T_8 (9 min) (3.33%). The maximum bacterial contamination % was recorded at T_3 (4 min)(20.00%) and the minimum was at T_6 (7 min) with (3.33%). The explants that did not survive the whole process were seen maximum in T_8 (9 min) (30.00%) with a minimum death % in T_2 (3 min), T_3(4 min) and T_4(5 min) (3.33%). In aseptic culture maximum % was recorded at T_6 (7 min) with (93.34%) and a minimum at T_1 (tap water) with (60.00%). The maximum survival % was recorded at T_4 (5 min)(83.33%) followed by T_5(6 min) (80.00%) and the minimum survival % was at T_1(Tap water) (56.67%). The increase in the exposure of timing to sterilants, lead to the death of explants which may be due to the heavy metal contamination of $HgCl_2$ proving phytotoxics that is against the survival of explants. Some explants in our experiment did not survive because of damage during sterilization procedure as $HgCl_2$ are toxic to the plant tissues, thus proper concentration and time duration of sterilizing agents should be carefully selected .Here it is observed that some bulb explants did not survive, it was probably due to the damage occurred during the sterilization procedure. The sterilizing agent should be used for an appropriate duration to control contamination. However, $HgCl_2$, which has mainly anti-bacterial action, was more efficient and showed more decontamination percentage. However, $HgCl_2$ was phytotoxic to hybrid Lilium bulb explants with the increase in the time duration[6].

Callus Induction

The sterilized bulb scale subjected to various treatments for callus emergence, development, proliferation and spread produced calli mass satisfactorily with the combination in MS media fortified with 0.5 mg/l BAP and 0.5 mg/l 2-4-D significantly induced callus in 16.33 DAI (Days after inoculation) followed by 0.5 mg/l BAP and 1.0 mg/l 2-4-D (18.33) DAI. A high concentration of Cytokinin in the medium promotes abundant callus proliferation (Chawala, 2002). A combination of 1.0 mg/l BAP and 2.0 mg/l 2-4-D induced maximum percentage of callus (90.00%) followed by 0.5mg/l BAP and 2.5 mg/l 2-4-D (86.67). Minimum callus percentage was recorded at the control (40.00) without any plant bioregulator (Table.4). Callus spread maximum was recorded at 1.0 mg/l BAP and 1.5 mg/l 2-4-D at 30 days and minimum were recorded in the control. In case of 60 days similar trends were observed in the same treatment. The results are in alignment with the findings of Aswath and Choudhry, (2002)[7]; Patnaik and Beura, (2008)[8]; Kumar(2017)[9]due to the synergetic effect of BAP was significant in maximum callus production.

Table1

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

Sl. no	Treatments	Fungal %	Bacteria%	Death %	Aseptic %	Survival %
1	T_1 (Tap Water)	16.67(24.60)	6.66(15.54)	10.00(18.44)	76.67(61.56)	66.67(55.12)
2	T_2 (0.1% $HgCl_2$ for 3 min)	16.67(24.60)	3.33(10.95)	3.33(10.95)	80.00(63.44)	76.67(61.56)
3	T_3 (0.1% $HgCl_2$ for 4 min)	10.00(18.44)	6.67(15.66)	3.33(10.95)	83.00(65.65)	80.00(63.44)
4	T_4 (0.1% $HgCl_2$ for 5 min)	3.33(10.95)	0(2.5)	3.34(10.94)	96.67(80.49)	93.33(75.33)
5	T_5 (0.1% $HgCl_2$ for 6 min)	3.33(10.95)	0(2.5)	6.67(15.66)	96.67(80.49)	90.00(71.56)
6	T_6 (0.1% $HgCl_2$ for 7 min)	3.33(10.95)	0(2.5)	10.00(18.44)	96.67(80.49)	86.67(69.09)
7	T_7(0.1% $HgCl_2$ for 8 min)	3.33(10.95)	3.34(11.11)	20.00(26.56)	93.33(75.33)	73.33(59.07)
8	T_8 (0.1% $HgCl_2$ for 9 min)	3.33(10.95)	3.33(10.95)	23.34(29.14)	93.34(75.44)	70.00(56.79)
	SE(m)±	2.67	-	2.87	3.84	4.55
	CD at 5%	7.4	5.65	7.95	10.66	12.62

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

Sl. no	Treatments	Fungal %	Bacteria%	Death %	Aseptic %	Survival %
1	T_1 (Tap Water)	20.00(26.56)	13.33(21.66)	10.00(18.44)	66.67(55.12)	56.67(49.21)
2	T_2 (0.1% $HgCl_2$ for 3 min)	16.67(24.60)	13.33(21.66)	3.33(10.95)	70.00(56.79)	66.67(55.12)
3	T_3 (0.1% $HgCl_2$ for 4 min)	10.00(18.44)	16.67(24.60)	3.33(10.95)	73.33(59.07)	70.00(56.79)
4	T_4 (0.1% $HgCl_2$ for 5 min)	3.33(10.95)	6.67(15.66)	3.33(10.95)	90.00(71.56)	86.67(69.09)
5	T_5 (0.1% $HgCl_2$ for 6 min)	3.33(10.95)	6.67(15.66)	6.67(15.66)	90.00(71.56)	83.33(66.12)
6	T_6 (0.1% $HgCl_2$ for 7 min)	3.33(10.95)	3.33(10.95)	13.34(21.71)	93.34(75.44)	80.00(63.44)
7	T_7(0.1% $HgCl_2$ for 8 min)	3.33(10.95)	10.00(18.44)	20.00(26.56)	86.67(69.09)	66.67(55.12)
8	T_8 (0.1% $HgCl_2$ for 9 min)	3.33(10.95)	6.67(15.66)	30.00(33.21)	90.00(71.56)	60.00(50.77)
	SE(m)±	2.37	-	2.80	2.43	3.44
	CD at 5%	6.58	7.72	7.77	6.74	9.54

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

Sl. no	Treatments	Fungal %	Bacteria%	Death %	Aseptic %	Survival %
1	T_1 (Tap Water)	20.00(26.56)	13.33(21.63)	10.00(18.44)	60.00(50.77)	56.67(49.21)
2	T_2 (0.1% $HgCl_2$ for 3 min)	20.00(26.56)	13.34(21.71)	3.33(10.95)	66.66(55.06)	63.33(54.51)
3	T_3 (0.1% $HgCl_2$ for 4 min)	16.67(24.60)	20.00(26.56)	3.33(10.95)	63.33(52.89)	60.00(50.77)
4	T_4 (0.1% $HgCl_2$ for 5 min)	6.67(15.66)	6.67(15.66)	3.33(10.95)	86.66(69.01)	83.33(66.12)
5	T_5 (0.1% $HgCl_2$ for 6 min)	3.33(10.95)	6.67(15.66)	10.00(18.44)	90.00(71.56)	80.00(63.44)
6	T_6 (0.1% $HgCl_2$ for 7 min)	3.33(10.95)	3.33(10.95)	16.67(24.60)	93.34(75.44)	76.67(61.56)
7	T_7(0.1% $HgCl_2$ for 8 min)	3.33(10.95)	10.00(18.44)	26.67(31.47)	86.67(69.09)	60.00(50.77)
8	T_8 (0.1% $HgCl_2$ for 9 min)	3.33(10.95)	6.67(15.66)	30.00(33.21)	90.00(71.56)	60.00(50.77)
	SE(m) ±	2.67	3.27	3.14	3.44	5.16
	CD at 5%	7.40	9.08	8.72	9.54	14.31

Table 4
Effect of Plant bioregulators on callus emergence, development, nature of callus, callus spread and callus colour of bulb scale explants.

Characters →			Culture-1(30 days)				Subculture-I	(30 Days)
Treatments ↓	BAP	2,4-D	Days to callus initiation	% of callus produced	Callus spread (cm)	Callus spread (cm)	Colour of callus	Nature of callus
T_1	-	-	20.00	40.00(39.23)	0.1×0.18	0.1×0.25	Light green	Compact
T_2	-	0.5	20.33	53.33(43.07)	0.1×0.21	0.1×0.29	Light green	Compact
T_3	-	1.0	20.66	50.00(45.00)	0.1×0.25	0.1×0.36	Light green	Compact
T_4	-	1.5	21.33	53.33(43.07)	0.1×0.26	0.2×0.40	Light green	Compact
T_5	-	2.0	22.00	73.33(59.07)	0.1×0.28	0.2×0.53	Light green	Compact
T_6	-	2.5	24.00	66.67(55.12)	0.1×0.26	0.2×0.56	Green	Compact
T_7	0.5	0.5	16.33	60.00(50.77)	0.1×0.18	0.1×0.51	Green	Compact
T_8	0.5	1.0	18.33	73.33(59.07)	0.1×0.21	0.1×0.82	Green	Compact
T_9	0.5	1.5	18.66	76.67(61.56)	0.1×0.24	0.2×0.81	Green	Compact
T_{10}	0.5	2.0	20.00	86.67(68.09)	0.1×0.33	0.2×0.62	Green	Compact
T_{11}	0.5	2.5	19.33	80.00(63.44)	0.1×0.30	0.2×0.44	Green	Compact
T_{12}	1.0	0.5	21.66	46.66(43.41)	0.1×0.26	0.2×0.40	Green	Compact
T_{13}	1.0	1.0	22.00	53.33(47.07)	0.1×0.32	0.3×0.26	Green	Compact
T_{14}	1.0	1.5	23.33	76.67(61.56)	0.1×0.48	0.3×0.86	Green	Compact
T_{15}	1.0	2.0	24.00	90.00(71.56)	0.1×0.45	0.5×0.50	Green	Compact
T_{16}	1.0	2.5	23.66	86.67(69.09)	0.1×0.44	0.4×0.71	Green	Compact
SE(m) ±			0.26	2.76				
CD at 5%			0.74	7.81				

CONCLUSION

It was concluded that hybrid Lilium Cv. Fangio bulb scale explants can be used to produce callus *in vitro* which could in turn be used to produce healthy plantlets under the aseptic condition on large a number and less time. Hence this investigation opens the way for use of biotechnology in mass propagation and improvement of hybrid Lilium Cv. Fangio.

CONFLICT OF INTEREST

Conflict of Interest declared none.

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We sincerely thank the above reviewers for peer reviewing the manuscript