The Bioscan 12(2): 765-770, 2017
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

EFFECT OF MEDIA AND DAYS AFTER POLLINATION ON EMBRYO GERMINATION IN CITRUS ROOTSTOCKS HYBRIDS

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KEYWORDS
Embryo rescue
Malt extract
Gibberellic acid
Sucrose
Rootstock
Rough lemon

Received on :
19.02.2017

Accepted on :
21.04.2017

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ABSTRACT
The present investigations were conducted to standerize the protocol for embryo rescue in new citrus rootstock hybrids. Rough lemon was crossed with three citrus rootstocks viz. rangpur lime (*Citrus limonia* Lush.), Carrizo citrange [*Citrus sinensis*(*L.*) Osbeck.x *Poncirus 0trifoliata* (*L.*) Raf] and trifoliate orange [*Poncirus trifoliata* (*L.*) Raf]. The embryos from hybrid fruits were rescued at 80, 90, 100, 110 days after pollination and cultured *in vitro*. The highest embryo germination 80.71% was observed when embryos were rescued 100 days after pollination which was followed by 77.53% those rescued after 110 days of pollination. Time taken for embryo germination in different hybrids ranged between 5.51 to 6.63 days, irrespective of the culture medium. There was no significant effect of pollen parents on shoot length, root length, number of leaves and number of roots per plantlet in all the cross combinations. Addition of malt extract, gibberellic and sucrose in culture medium improved the embryo germination and embling growth. The highest 68.65% and earliest embryo germination and better growth performance of emblings were recorded when the embryos were cultured after 100 days of pollination on MS medium supplemented with malt extract (0.5 g/L) + sucrose (50 g/L), irrespective of the pollen parent.

INTRODUCTION

Citrus has a prominent place among fruits and is being grown extensively all over the world. In India, it ranks third after mango and banana covering an area of 1.08 m ha with annual production of 11.15 million tonnes (Anon, 2014). India ranks sixth among citrus producing countries of the world contributing 5.8 per cent of total citrus production. Still India is far behind because of lower productivity per unit area. In Punjab, it ranks first among all the fruits with an acreage of 50, 428 ha and 10, 44, 202 metric tonnes production (Anon, 2015). Among the citrus fruits, Kinnow mandarin is the major fruit of Punjab having an area of 47, 101 ha with an annual production of 10, 17, 725 MT. The main reasons of low productivity are *Phythophthora* root rot and citrus decline,. Which are mainly influenced by rootstocks because rootstock is a major contributor to tree performance and longevity, as it determines tolerance to various biotic and abiotic stresses. Novel method of combating *Phytophthora* disease is the use of tolerant and resistant rootstock. In India, particularly in Punjab citrus orchards are established mainly on rough lemon rootstock. Although, this rootstock has well adapted under Punjab conditions, but its susceptibility to *Phytophthora* fungus has become a major cause of citrus decline and overall citrus production. It is difficult to replace this rootstock considering its resistance to *Tristeza* virus, suitability to high pH soil of North Western India and high yields obtained in most of scion varieties budded on it. Although many citrus rootstocks have been reported to be resistant or tolerant to *Phytophthora* but, none of these rootsocks are suitable for Kinnow mandarin under North Western condition. Hence, there is an urgent need to develop *Phytophthora* tolerant/ resistant rootstock of citrus suitable for Kinnow mandarin by using rough lemon as one of the parents.

Because the production of large number of polyembryonic seeds with high germination rate and percentage of nucellar progenies is one of the essential attributes that a rootstock should possess (Broadbent and Gollnow, 1993). This is because nucellar embryos derived from somatic cells during the early stage of seed development, are genetically identical to their mother plant and can thus ensure the uniformity of the nursery seedlings.

However this polyembryony is one of the main hurdle in citrus breeding because zygotic embryos have difficulty in surviving since they have to compete for nutrients and space with the nucellar embryos that develop from nucellar tissues (Soost and Roose, 1996). The size and survival of sexual embryos were inversely related to the number of embryos per seed. The nucellar embryo limits the range of genetic variability that can be observed in the progeny of a cross and thus the possibility of finding new genotypes. The success of embryo culture is determined by the stage of embryo rescue, the composition of medium and the genotype of the plant (Yi *et al.,* 1998). The younger embryos are the most difficult to rescue *in vitro* and at later stages the seeds have developed too much

to separate the embryos. The nucellar embryos had not been found in the younger seeds while the zygotic embryo had already reached the heart shaped stage (Rangann *et al*., 1969). Hence, embryo culture could avoid screening of nucellar and zygotic seedlings in polyembryonic cultivars. The largest embryo presumed to be zygotic and nucellar embryos are smaller at young age

Embryo rescue continues to be a very useful tool to overcome the problem of embryo abortion in citrus sexual crosses and many triploid seedlings have been obtained by interploid crosses using this technique (Yi and Deng, 2001; Jaskani *et al*., 2005 and Viloria *et al*., 2005, Soost and Roose, 1996). The success of embryo rescue depends on ingredient of medium and embryo developing stages (Jaskani *et al*. 2005). The germination capacity of citrus embryos can be affected by embryo's genetic structure and embryo developing stage (Viloria *et al*. 2005). Various studies have reported that the highest germination rate in embryo development stages were obtained 115 days after pollination in two hybridization combinations. Similarly, 118 days after pollination for embryo germination has been reported by (Chagas *et al*. 2005). On the other hand, there were reports indicating good embryo germinations after 50 days (Wang *et al*., 1999), 80 days (Tan *et al*., 2007), 100 days (Deng *et al*., 1996), 105 days (Scarano *et al*., 2003) and 120 days (Carimi *et al*., 1998; Das *et al*., 2000; Kurt and Ulger 2014) in different citrus genotypes. The culture media also affect the germination and growth of embryo as described by the addition of 0.01 mg/L GA3 (Chagas *et al*., 2005), 1 mg/L GA3 (Ollitrault *et al*., 2007), and 2 mg/L GA3 (Gmitter *et al*., 1990) in growing media for embryos is important in developing citrus plan. Therefore present studies were conducted to optimize embryo rescue and germination techniques for higher recovery of citrus rootstock hybrids of Rough lemon as affected by different genotypes used as male parent.

MATERIALS AND METHODS

The present investigations were carried out at Rough Lemon Rootstock Block, College Orchard and Tissue Culture Laboratory, Department of Fruit Science, PAU, Ludhiana. Rough lemon was crossed with rangpur lime (*Citrus limonia* Lush.), Carrizo citrange [*Citrus sinensis*(*L*.) Osbeck.x *Poncirus trifoliata* (*L*.) Raf] and trifoliate orange [*Poncirus trifoliata* (*L*.)Raf] as pollen parent. The developing fruits were harvested at 80, 90, 100 and 110 days after pollination for the embryo rescue studies. After harvesting the fruits were brought to the Tissue Culture Laboratory where the fruits were washed with Teepol solution (0.1%) followed by three washing with running tap water. The fruits were surface sterilized with mercuric chloride alone or along with 70 per cent ethanol for different exposure durations (2.5 to 10 min) as presented in Table 1. Further, in some treatments after mercuric chloride treatment for 2.5 to 10 minutes the fruits were dipped in 70 per cent ethanol and placed on flame for a while. After surface sterilization the fruits were cut by sharp blade at the equatorial zone avoiding the core where seeds are embedded under a Laminar Air Flow Cabinet. The both halves of the fruit were twisted in opposite direction until their total separation (Jaskani M *et al*., 2005) . The seeds from the fruits were collected in petri plates and embryo excision was performed under 100X magnification using a stereomicroscope. The immature embryos were carefully excised and categorized into two groups viz. small embryos (globular, heart and torpedo-shaped) and cotyledonary embryos (developing embryos but not mature). The excised embryos were cultured on Murashiage and Skoog (MS) medium fortified with variable levels of malt extract, $GA_3$and sucrose (Table 2) under aseptic conditions to induce germination (Thakur B and Singh H, 2015) . One embryo was cultured in a test tube and 3-4 embryos were cultured in petriplates (Tan M *et al*., 2007). The pH of the medium was adjusted to 5.8 with pH meter using 1 N HCl or 1 N NaOH before adding agar (7.5g/L) as solidifying agent in the medium. The cultured embryos in culture vessels were maintained at $25 \pm 2°C$ with 16 hours of continuous fluorescent light (2,000 lux) followed by a dark period of 8 hours in an incubation room. The incubation temperature and photoperiod were similar in all the experiments (Turgutoglu *et al*., 2015). The data were recorded on the per cent contamination, per cent germination, days taken for embryo germination and growth parameters of emblings after six weeks of inoculation. The experiment was conducted as per completely randomized design with three replications per treatment where ten cultures formed one replication. Statistical analysis was done using CPCS-1 software package developed at Punjab Agricultural University by (Cheema and Singh, 1990).

RESULTS AND DISCUSSION

There was significant reduction in per cent contamination with an increase in duration of exposure to 0.1 per cent $HgCl_2$ (Table 1). Among various treatments, 10 minute dip in $HgCl_2$ (0.1%) followed by ethanol (70%) dip and exposure to flame was found to be the best surface sterilization treatment with 93.34 per cent healthy embryos. It was at par with 7.5 minutes exposure to $HgCl_2$ (0.1%) where 92.14 percent healthy embryo

Table 1: Effect of surface sterilant treatment on contamination and embryo survival

Time of exposure to $HgCl_2$(min)	Contamination (%)					Embryo Survival (%)				
	$HgCl_2$ alone	$HgCl_2$ + 2minethanol	$HgCl_2$ + 5 minethanol	$HgCl_2$ + flame	Mean	$HgCl_2$ alone	$HgCl_2$ + 2 minethanol	$HgCl_2$ + 5min ethanol	$HgCl_2$ + flame	Mean
2.5	41.07	37.93	26.83	16.40	30.56	58.94	62.26	68.45	83.60	68.31
5.0	33.01	28.73	19.10	12.45	23.32	67.00	71.70	74.21	87.02	74.98
7.5	23.51	20.94	14.30	7.77	16.63	76.50	79.22	80.67	92.14	82.13
10.0	22.44	19.19	13.42	7.04	15.52	77.56	80.52	79.96	93.34	82.85
Mean	30.01	26.70	18.41	10.91		70.00	73.43	75.82	89.03	
$LSD_{(0.05)}$	2.44						7.56			

Table 2: Effect of fruit development stage and medium composition on *in vitro* per cent embryo germination of different citrus rootstock hybrids

Media/Embryo age	Rough lemon x Rangpur lime Days after pollination				Mean	Rough lemon x Trifoliate orange Days after pollination				Mean	Rough lemon x Carrizo citrange Days after pollination				Mean
	80	90	100	110		80	90	100	110		80	90	100	110	
M_1-MS + GA_3 (0.5 mg/L) + sucrose (30 g/L)	30.33	44.78	74.02	70.85	54.99	29.48	42.27	72.34	68.27	53.09	32.48	45.67	75.02	71.67	56.21
M_2-MS + GA_3 (1.0 mg/L) + sucrose (30 g/L)	35.5	47	77.29	74.02	58.45	35.35	48.53	78.95	74.02	59.21	33.34	46.67	76.09	72.98	57.27
M_3-MS + GA_3 (1.5 mg/L) + sucrose (30 g/L)	33.27	45.44	75.4	71.67	56.45	33.27	46.67	76.56	72.34	57.21	31.6	44.78	74.01	70.67	55.27
M_4-MS + GA_3 (0.5 mg/L) + sucrose (50 g/L)	37.35	49.5	79.56	76.35	60.69	35.48	48.58	78.4	74.67	59.28	37.78	50.34	80.84	76.53	61.37
M_5-MS + GA_3 (1.0 mg/L)+ sucrose (50 g/L)	42.02	54.62	84.12	80.95	65.43	42.02	55.95	85.09	81.62	66.17	39.67	52.2	82.37	78.02	63.06
M_6-MS + GA_3 (1.5 mg/L) + sucrose (50 g/L)	39.67	52.6	82.29	79.56	63.53	37.78	50.34	80.95	76.53	61.4	36.27	49	79.39	75.87	60.13
M_7-MS + malt extract (0.25 g/L) + sucrose (50 g/L)	37.78	49.67	79.56	76.53	60.88	37.91	50.53	80.22	76.64	61.33	38.67	52.03	81.95	77.02	62.42
M_8-MS + malt extract (0.5 g/L) + sucrose (50 g/L)	45.29	57.62	87.57	84.12	68.65	43.02	56.95	85.22	82.29	66.87	42.2	55.84	85.22	81.95	66.3
M_9-MS + malt extract (0.75 g/L) + sucrose (50 g/L)	44.56	56.95	86.6	83.76	67.97	44.56	57.29	87.24	83.76	68.21	43.02	56.95	86.91	82.29	67.29
Mean	38.42	50.91	80.71	77.53		37.65	50.79	80.55	76.68		37.22	50.39	80.2	76.33	
$LSD_{(0.05)}$	DAP = 3.10					DAP = 3.16						DAP = 3.52			
	Media = 4.65					Media = 4.74						Media = 5.28			
	DAP x Media = NS					DAP x Media = NS						DAP x Media = NS			

were obtained. It was followed by 5 minutes dip in $HgCl_2$ (0.1%) and exposure to flame after dipping in ethanol (70%). The minimum healthy embryos were recovered with 2.5 minute exposure to $HgCl_2$ (0.1%) alone. The embryo germination was significantly affected by the age of embryo and it increased with the increase in fruit development stage up to 100 days after pollination in all crosses, the embryos from rough lemon x rangpur lime, rough lemon x trifoliate orange and rough lemon x Carrizo citrange crosses exhibited significantly higher embryo germination (80.71, 80.55 and 80.20 %, respectively) at 100 days after crossing and minimum embryo germination (38.42, 37.65 and 37.32 %, respectively) was recorded after 80 days. The poor embryo germination after 80 days of crossing indicates that at this stage embryos were immature to germinate. Similarly, (Kurt and Ulger, 2014) found that the embryos harvested at 80 days showed 32 per cent embryo germination which increased with the increase in age of harvested embryos and per cent embryo germination was observed from embryos excised 120 days after pollination. Embryo immaturity was probably the reason for the poor germination at earlier stage i.e. 80 days after crossing (Theobald and Hough, 1960). Generally, young citrus embryos excised from polyembryonic seeds at around 100 days after pollination showed better recovery of emblings (Deng *et al*., 1996 and Tusa *et al*., 1996). Depending upon the genotype of the citrus the suitable stage for embryo rescue operations was found to be 50 days (Wang *et al*., 1999), 80days (Tan *et al*., 2007), 100 days (Tusa *et al*., 1996), 105 days (Scarano *et al*., 2005), 118 days (Chagas *et al*., 2005) and 135-150 days (Perez Tornero and Porras, 2008) after pollination.

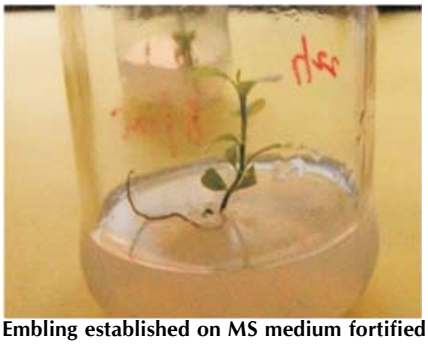
The embryo germination in all the three cross combinations was significantly affected by culture medium. In rough lemon x rangpur lime cross combination, maximum germination (68.65%) was recorded with MS medium supplemented with 0.5 g/L Malt extract (ME) and 50g/L sucrose (M_8). The minimum germination (54.99%) was observed with M_1 medium-MS medium supplemented with GA_3 (0.5g/L) and sucrose (30g/L). However, the hybrids from rough lemon x trifoliate orange and rough lemon x Carrizo citrange had maximum embryo germination with MS medium supplemented with ME (0.75 g/L) and sucrose (50 g/L), irrespective of days after pollination which was at par with M_8- MS + ME (0.5g/L) and sucrose (50g/L) and M_5 -MS + GA_3 (1.0 mg/L) + sucrose (50g/L). In both, rough lemon x rangpur lime and rough lemon x trifoliate orange cross combinations, minimum embryo germination was observed on M_1-MS + GA_3 (0.5 mg/L) + sucrose (30g/L), irrespective of stage of embryo development. Whereas in rough lemon x Carrizo citrange crosses the minimum embryo germination (55.27%) was observed on M_3-MS + GA_3 (1.5 mg/L) + sucrose (30g/L). In all the three cross combinations, the interaction between days after pollination and culture medium was found non significant. Likewise, (Carimi *et al*., 1998) also observed that early cotyledonary embryos responded better to MS medium with malt extract 0.5g/L and (Turgutoglu *et al*., 2015) found that best germination was observed in MS medium supplemented with GA_3 (2.0 mg/l).

The addition of gibberellic acid (GA_3) also affect the embryo germination and higher embryo germination was recorded with higher dose of GA_3 (1.0 mg/L) in comparison to lower

Table 3: Effect of pollen parent and medium composition on time for embryo germination

Media/ Hybrids	Rough lemon x Rangpur lime	Rough lemon x Trifoliate orange	Rough lemon x Carrizo citrange	Mean
M_1- MS + GA_3 (0.5 mg/L) + sucrose (30 g/L)	7.67	7.92	8.13	7.91
M_2- MS + GA_3 (1.0 mg/L) + sucrose (30 g/L)	6.01	7.15	7.73	6.96
M_3- MS + GA_3 (1.5 mg/L) + sucrose (30 g/L)	6.11	7.84	7.61	7.19
M_4-MS + GA_3 (0.5 mg/L) + sucrose (50 g/L)	5.92	7.8	6.44	6.72
M_5- MS + GA_3 (1.0 mg/L) + sucrose (50 g/L)	5.39	5.81	5.47	5.56
M_6-MS + GA_3 (1.5 mg/L) + sucrose (50 g/L)	5.07	6.42	5.87	5.79
M_7-MS + malt extract (0.25 g/L) + sucrose (50 g/L)	4.72	6.33	6.22	5.75
M_8-MS + malt extract (0.5 g/L) + sucrose (50 g/L)	4.52	4.93	4.87	4.77
M_9-MS + malt extract (0.75 g/L) + sucrose (50 g/L)	4.22	5.46	4.76	4.81
Mean	5.51	6.63	6.35	
$LSD_{(0.05)}$	Crosses = NS, Media = 1.84, Crosses x Media = NS			

Embryo culture on MS medium fortified with malt extract (0.5 g/L) + sucrose (50g/L)



Embling established on MS medium fortified with GA_3 (1.0 g/L) + sucrose (50g/L)

Embling on MS medium fortified with malt extract (0.5 g/L) + sucrose (50g/L)

Hardening and growth of emblings rescued on MS medium fortified with GA_3(1.0g/L) + sucrose (50g/L)

Hardening and growth of emblings rescued on MS fortified with malt extract (0.5 g/L) + sucrose (50g/L)

Plantlet transferred to pot after hardening

dose (0.5 mg/L), irrespective of sucrose level (Table 2). Similar results were reported by (Kurt and Ulger, 2014) who found that the embryo germination was maximum with GA_3 (1.0 mg/L) in hybrids of common sour citrange x Carrizo citrange at various developmental stages of embryo. The embryo germination was also affected by variation in sucrose concentration. The higher embryo germination was recorded with 50g/L of sucrose as compared to 30g/L, irrespective of GA_3 supplementation. Similarly, (Sykes and Lewis, 1996) found that increasing the sucrose concentration and addition of GA_3 enhanced embryo germination in Imperial and Silverhill satsuma mandarins. (Carimi *et al*., 1998) also observed that high sucrose concentration in culture medium was necessary for germination of pro-embryos in sour orange.

The culture medium also affected the time taken for embryo germination significantly. Time taken for embryo germination in different hybrids ranged between 5.51 to 6.63 days,. The data in Table 3 shows that in all the cross combinations, the culture medium M_8- MS + ME (0.5 g/L) + sucrose (50 g/L) resulted in earliest embryo germination (4.77 days) which was at par with M_9- MS + ME (0.75 g/L) + sucrose (50 g/L), M_5- MS + GA_3(1.0mg/L) and sucrose (50 g/L) and M_6- MS + GA_3 (1.5 mg/L) and sucrose (50 g/L).Maximum number of days taken for germination was recorded with M_1medium - MS + GA_3 (0.5 mg/L) and sucrose (30 g/L). There was no significant effect of pollen parents on shoot length, root length, number of leaves and number of roots per plantlet in all the cross combinations (Table 4). However, culture medium had a

Table 4: Effect of pollen parent and medium composition on emblinggrowth of different citrus rootstock hybrids

Media/ Hybrids	Shoot length				Root length				Number of leaves				Number of roots			
	R x R	R X T	R x C	Mean	R x R	R X T	R x C	Mean	R x R	R X T	R x C	Mean	R x R	R X T	R x C	Mean
M_1- MS + GA_3 (0.5 mg/L) + sucrose (30 g/L)	3.94	3.34	3.53	3.6	6.51	5.61	5.96	6.03	5.44	4.8	5.05	5.1	2.39	1.51	1.88	1.93
M_2- MS + GA_3 (1.0 mg/L) + sucrose (30 g/L)	4.66	3.73	4.34	4.25	7.48	6.4	6.73	6.87	6.39	5.48	5.86	5.91	2.82	2.14	2.59	2.51
M_3- MS + GA_3 (1.5 mg/L) + sucrose (30 g/L)	4.34	3.49	4.01	3.95	7.15	6.15	6.44	6.58	5.93	5.18	5.58	5.56	2.64	2.06	2.32	2.34
M_4-MS + GA_3 (0.5 mg/L) + sucrose (50 g/L)	4.11	3.47	3.73	3.77	6.93	5.86	6.25	6.35	5.77	4.99	5.4	5.38	2.48	1.87	2.1	2.15
M_5- MS + GA_3 (1.0 mg/L) + sucrose (50 g/L)	5.6	4.66	5	5.09	8.15	7.05	7.39	7.53	6.82	6.15	6.48	6.48	3.25	1.73	2.86	2.61
M_6-MS + GA_3 (1.5 mg/L) + sucrose (50 g/L)	4.48	4.01	4.43	4.31	7.63	6.51	6.93	7.02	6.39	5.88	5.94	6.07	2.69	2.49	2.35	2.51
M_7-MS + malt extract (0.25 g/L) + sucrose (50 g/L)	4.3	3.61	4.11	4.01	7.24	6.35	6.54	6.71	6.01	5.28	5.66	5.65	2.82	1.96	2.38	2.39
M_8-MS + malt extract (0.5 g/L) + sucrose (50 g/L)	6.82	6.15	6.49	6.49	9.53	8.45	8.92	8.97	8.31	7.52	7.92	7.92	3.45	2.19	3.28	2.98
M_9-MS + malt extract (0.75 g/L) + sucrose (50 g/L)	6.7	6.17	6.55	6.48	9.31	8.79	9.12	9.07	8.53	7.65	7.93	8.04	3.57	2.93	3.33	3.28
Mean	4.99	4.29	4.69		7.77	6.8	7.14		6.62	5.88	6.2		2.90	2.10	2.56	
$LSD_{0.05}$	Crosses = NS				Crosses = NS				Crosses = NS				Crosses = NS			
	Media = 2.17				Media = 2.10				Media = NS				Media = NS			
	Crosses x Media = NS				Crosses x Media = NS				Crosses x Media = NS				Crosses x Media = NS			

R x R (Rough lemon x Rangpur lime); R X T (Rough lemon x Trifoliate orange); R x C (Rough lemon x Carrizo citrange)

significant role in root and shoot length and the emblings had the longest shoots (6.49 cm) on M_8 - MS medium supplemented with 0.5g/LME and 50 g/L sucrose (Fig.1.). The shortest shoot length (3.60 cm) was recorded in M_1 - MS + GA_3 (0.5 mg/L) + sucrose (30g/L). Ram *et al* (10) observed that rooting and plantlet establishment were at IBA (3.0 mg/L) with maximum survival rate (95%).

Similarly, the root length was also found to be affected by culture medium. The maximum root length of 9.07 cm was obtained with M_9 - MS + ME (0.75 g/L) + sucrose (50g/L) whereas, the minimum root length was observed in M_1 - MS + GA_3 (0.5 mg/L) + sucrose (30g/L), irrespective of cross combinations. However, both the medium as well as pollen parent had no significant effect on number of leaves and roots per plantlet. Similarly, (Perez Tornero and Porras, 2008) observed variation of shoot and root length of embryoids from different species with four different media.

In the present investigations, it was concluded that maximum and earliest embryo germination and better growth performance of emblings were recorded when the embryos were cultured after 100 days of pollination on medium M_8- MS medium supplemented with ME (0.5 g/L) + sucrose (50 g/L), irrespective of the pollen parent.

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