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Research Article

Establishing surface sterilization protocol for clonal apple rootstock MM106

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Abstract
Surface sterilization is crucial in preparing viable and uncontaminated explants for tissue culture. Utilizing the right sterilizing agent in this procedure can efficiently eliminate the majority of surface contaminants. The present study aimed to establish the best sterilization procedure for clonal apple rootstock MM106 using shoot tips and nodal segments. Two important sterilizing agents, mercuric chloride (ranging from 0.05% to 0.3%) for various durations (2 to 6 minutes) and sodium hypochlorite (ranging from 3.0% to 12%) for different durations (10 to 30 minutes), were employed. The results indicated that the maximum aseptic conditions (80.00% and 80.67%) and survival rates of the cultures (60.66% and 67.33%) for shoot tips and nodal segments, respectively, were achieved through surface sterilization with 0.1% mercuric chloride for 4 minutes, specifically during April, in comparison to March and May of 2021 due to low phenol content and high meristematic activity. The results yielded valuable insights for efficiently propagating clonal apple rootstocks on a local scale, and they have the potential to offer guidance for establishing commercial facilities dedicated to producing these specific clonal apple rootstocks and varieties.

Keywords: Apple, Clonal rootstock, Explants, Surface sterilization, MM106

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INTRODUCTION

Aside from soil and dirt particles, vegetative shoots of fruit plants gathered from open fields for explant purposes also contain fungi and bacteria. These microorganisms could pose significant challenges to in vitro propagation (Wolella, 2017). Consequently, it is imperative to meticulously and effectively surface sterilize explants before initiating cultivation. Since bacteria and fungi have the potential to infect the plant culture and inflict continuous damage throughout the cultivation phase, sterilization becomes indispensable. Thus, to economize time and effort, adopting a precise and thorough sterilization approach is crucial when employing tissue culture techniques (Lal *et al*., 2023). To minimize contamination, optimizing the concentrations of sterilizing agents becomes crucial. It is essential to determine the appropriate concentration and exposure time for these agents to enhance the success rate of in vitro cultures, as an incorrect concentration could impede cell division and growth. The literature documents the use of various agents such as ethanol, sodium hypochlorite (NaOCl), calcium chloride ($CaCl_2$), hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$), and bromine water for disinfecting the surface contamination in explants (Lal *et al*., 2023 and Dalal *et al*., 2006). Despite its severe effects, mercuric chloride ($HgCl_2$) is commonly used to mitigate microbial contamination for surface sterilization, particularly in apple cultivars and rootstock tissue culture. The present study aimed to establish the most viable and effective protocol for surface sterilization of clonal apple rootstock MM106, using shoot tips and nodal explants, to facilitate in vitro propagation.

MATERIALS AND METHODS

Plant material

About 10-15 cm actively growing shoots of clonal apple rootstock MM106 were removed using sterile scissors from the 2-3-year-old mother block established at Regional Horticulture Research Sub-Station (RHRSS), Bhaderwah-Jammu (J&K) during their active growth phase in March, April, and May of 2021. These shoots were brought to the Plant Tissue Culture Laboratory, Udheywala and Plant Tissue Culture Laboratory, School of Biotechnology of Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (J&K) for explants preparation.

Preparation and sterilization of explants

Explants were taken from the shoot tip (0.5 cm to 0.75 cm) and nodal segment (0.5 cm to 0.75 cm). The explants were treated for 20 minutes with 0.2% Bavistin solution, then for varied periods with $HgCl_2$ (0.05%, 0.1%, and 0.2%) and NaOCl (3.0%, 8.0%, and 12.0%), respectively, for 2, 4, and 6 minutes and 10, 20 and 30 minutes. Explants were rinsed three to four times in sterile (autoclaved) distilled water after surface sterilization to get rid of any lingering sterilant (Modgil *et al*., 2009). The cultures were continuously examined for 10 -15 days after inoculation to determine the aseptic and survival percentage. After the examination the cultures that remained unaffected microbial infection and showed little growth were considered for culture establishment.

Statistical analysis

The data generated was subjected to a completely Randomized Design analysis, as defined by Singh *et al*. (1998), to assess the outcomes of numerous experiments.

RESULTS

Sterilization of the material

The clonal apple rootstock MM106 explants were procured from an open field during March, April, and May of 2021, which contained adherent soil and dirt particles, fungi, and bacteria. Therefore, before cultivating,

Table 1. Different sterilization treatments on shoot tips and nodal segments of clonal apple rootstock MM106

Treatments	Concentration	Time of Exposure	Treatments	Concentration	Time of Exposure
Control	-	-	Control	-	-
$HgCl_2$	0.05%	2 minutes	NaOCl	3%	10 minutes
$HgCl_2$	0.05%	4 minutes	NaOCl	8%	20 minutes
$HgCl_2$	0.05%	6 minutes	NaOCl	12%	30 minutes
$HgCl_2$	0.2%	2 minutes	NaOCl	3%	10 minutes
$HgCl_2$	0.2%	4 minutes	NaOCl	8%	20 minutes
$HgCl_2$	0.2%	6 minutes	NaOCl	12%	30 minutes
$HgCl_2$	0.3%	2 minutes	NaOCl	3%	10 minutes
$HgCl_2$	0.3%	4 minutes	NaOCl	8%	20 minutes
$HgCl_2$	0.3%	6 minutes	NaOCl	12%	30 minutes

a thorough and efficient surface sterilization was required. In terms of aseptic cultures and culture survival, explants treated with $HgCl_2$ outperformed NaOCl (Table 3). Maximum aseptic cultures (83.33%) were obtained after treatment with 0.1% $HgCl_2$ for 4 minutes, significantly superior to all other treatments (Fig. 1). Treatment of 0.1% $HgCl_2$ for 6 minutes showed 70.00% aseptic cultures followed by 0.1% $HgCl_2$ for 2 minutes (63.33%), and 0.2% $HgCl_2$ for 2 minutes (55.00%), while control had the lowest aseptic cultures (0.00%) (excluding sterilising agents). The nodal segment produced 49.0% aseptic cultures between the two explants, significantly more than the yield of shoot tip explant**s** (41.0%) (Table 2). The interaction results showed that shoot tips recorded 80.00% aseptic cultures, followed by 66.67% with 0.1% $HgCl_2$ after 6 minutes, while nodal segment explants gave the highest percentage of aseptic cultures (86.67%), followed by 73.33% with 0.1% $HgCl_2$ for 4 minutes. However, for both explants, the lowest aseptic cultures (0.00%) were noted under control.

The data obtained concerning the effect of mercuric chloride and sodium hypochlorite on the percent survival of cultures during all the months of the study showed a significant effect on percent survival of cultures of clonal apple rootstocks MM106 (Table 4). Comparing both sterilizing agents, mercuric chloride gave better results than sodium hypochlorite. 0.1% $HgCl_2$ for 4 minutes gave maximum survival of cultures (64.00%), which was significantly higher among all other treatments, followed by 0.1% $HgCl_2$ for 2 minutes (39.67%), 0.1% $HgCl_2$ for 6 minutes (38.33%) and 0.05% $HgCl_2$ for 6 minutes (21.00%). The minimum survival of cultures (0.00%) was observed under control (Fig. 2) as compared to 64.00 percent with 0.1% $HgCl_2$ for 4 minutes. Between the explants, the highest survival of cultures (27.80%) was recorded by nodal segment, which was significantly higher than the shoot tip (21.50%). The interaction data indicated that treating nodal segments with 0.1% $HgCl_2$ for 4 minutes gave maximum survival of cultures (67.33%) followed by 0.1% $HgCl_2$ for 6 minutes (45.00%), while shoot tip gave (60.66%) survival of cultures followed by 0.1% $HgCl_2$ for 2 minutes (34.66%). However, the lowest survival of cultures (0.00%) was recorded under control for both the explants.

DISCUSSION

In the present study, shoot tip and nodal segment explants from actively growing shoots of MM106 were used to establish the most viable methodology for surface sterilization of the clonal apple rootstock MM106. Explants obtained from an open field contain fungi in

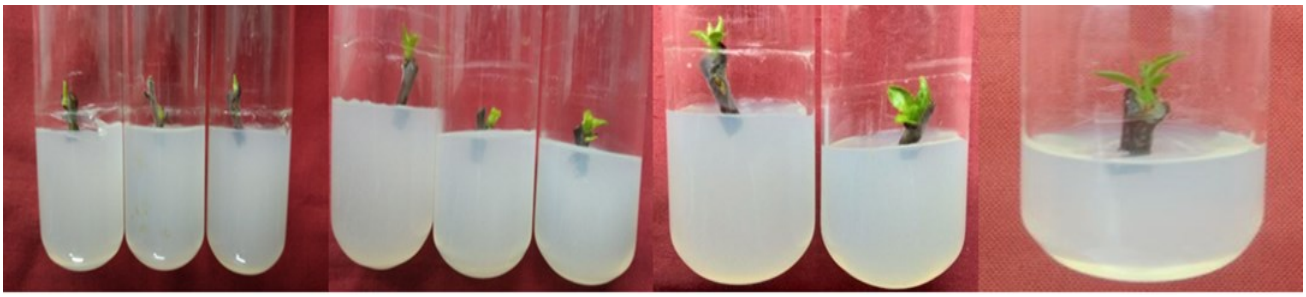

Fig. 1. *Explants treated with 0.1% $HgCl_2$ for 4 minutes showing maximum aseptic as well as survival of cultures*

Table 2. Effect of mercuric chloride ($HgCl_2$) on per cent aseptic cultures in apple clonal rootstocks during April of 2021

Treatment	Duration (min.)	MM106		
		Shoot tip	Nodal segment	Mean
Control	-	0.00(0.00)*	0.00(0.00)	0.00(0.00)
$HgCl_2$ (0.05%)	2	26.67(30.98)	40.00(39.21)	33.33(35.09)
$HgCl_2$ (0.05%)	4	30.00(33.19)	40.00(39.21)	35.00(36.20)
$HgCl_2$(0.05%)	6	30.00(33.19)	43.33(41.13)	36.67(37.16)
$HgCl_2$ (0.1%)	2	63.33(52.75)	63.33(52.75)	63.33(52.75)
$HgCl_2$ (0.1%)	4	80.00(63.40)	86.67(68.82)	83.33(66.11)
$HgCl_2$ (0.1%)	6	66.67(54.76)	73.33(58.98)	70.00(56.87)
$HgCl_2$ (0.2%)	2	53.33(46.90)	56.67(48.82)	55.00(47.86)
$HgCl_2$ (0.2%)	4	33.33(35.20)	50.00(44.98)	41.67(40.09)
$HgCl_2$(0.2%)	6	26.67(30.98)	36.67(37.21)	31.67(34.09)
Mean		41.00(38.13)	49.00(43.11)	
$CD_{(0.05)}$	Explant (E) = 1.47 Sterilant (S) = 3.29 E × S = 4.66			

*Arcsine transformed values in parenthesis

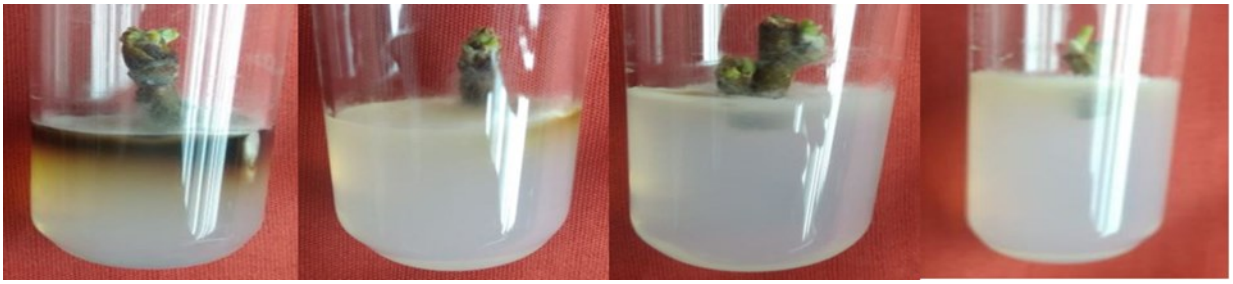

Fig. 2. *Explants treated with the control showing no aseptic and survival of cultures*

addition to soil and dirt particles that have become attached. Therefore, explants must be thoroughly and effectively surface sterilized before being cultured. The creation of aseptic cultures is the first crucial stage in the *in vitro* propagation process, as highlighted by (Lal *et al*., 2023). According to Jaime *et al*. (2019), the better establishment of explants on apple trees depends heavily on the surface sterilization of the explants with mercuric chloride. The season and kind of explant**s**, in addition to other parameters, have been shown by Murashige (1974) to affect the effectiveness of explant *in vitro* regeneration. The month in which clonal apple rootstocks are cultured affects their potential to regenerate, with explants at an active growth stage (summer months) having the highest capacity to do so (Soni *et al*., 2010).

In the present work, explants treated with 0.1% $HgCl_2$ for 4 minutes provided more favourable outcomes than NaOCl regarding uncontaminated and culture survival. Nodal segments between two separate explants provided the highest percentage of aseptic and culture survival in April compared to March and May. This is because, during the month of April, the content of phenols in apple plant shoots is very low and meristematic activity is very high (Lal *et al*., 2021). However, the physiological stage of the mother plant at the moment of excision significantly impacts how well an isolated plant material is disinfected (Kaushal *et al*., 2005). Increasing the concentration and duration of exposure to surface sterilant decreased the survival rate of cultures**,** while increasing the number of aseptic cultures. This might be due to the phytotoxicity that $HgCl_2$ causes in plants (Wolella, 2017; Rattanpal *et al*., 2011). Optimum concentration and exposure time are essential for increasing the success rate of *in vitro* culture**s** because the inaccurate concentration will limit cell division and further growth of explants (Hashim *et al*. 2021). Numerous studies have also shown that decreasing the concentration and duration of surface sterilants like $HgCl_2$ affected the percentage of cultures that survived (Ghanbari *et al*., 2014).

Conclusion

The present study developed a protocol for surface sterilization of clonal apple rootstock MM106 using shoot tip and nodal segment explants. During sterilization protocol establishment, the highest significant survival and aseptic values (83.33% and 64.00%) were recorded when explants were surface sterilized with

Table 3. Effect of sodium hypochlorite (NaOCl) on per cent aseptic cultures in apple clonal rootstocks during April of 2021

Treatment	Duration (min.)	MM106		
		Shoot tip	Nodal segment	Mean
Control	-	0.00(0.00)*	0.00(0.00)	0.00(0.00)
NaOCl (3%)	10	23.33(28.76)	30.00(30.87)	26.67(30.87)
NaOCl (3%)	20	30.00(33.19)	43.33(37.16)	36.67(37.16)
NaOCl (3%)	30	30.00(33.19)	53.33(40.05)	41.67(40.05)
NaOCl (8%)	10	43.33'(41.13)	60.00(45.98)	51.67(45.98)
NaOCl (8%)	20	63.33(52.75)	76.67(56.97)	70.00(56.97)
NaOCl (8%)	30	63.33(52.75)	66.67(53.75)	65.00(53.75)
NaOCl (12%)	10	60.00(50.83)	60.00(50.79)	60.00(50.79)
NaOCl (12%)	20	53.33(46.90)	53.33(46.90)	53.33(46.90)
NaOCl (12%)	30	20.00(26.06)	33.33(30.63)	26.67(30.63)
Mean		38.67(36.56)	47.67(42.06)	
$CD_{(0.05)}$	Explant (E) = 2.02 Sterilant(S) = 4.51 E × S = 6.38			

*Arcsine transformed values in parenthesis

Table 4. Effect of mercuric chloride ($HgCl_2$) on per cent survival cultures in apple clonal rootstocks during April of 2021

Treatment	Duration	MM106		
		Shoot tip	Nodal segment	Mean
Control	-	0.00(0.00)*	0.00(0.00)	0.00(0.00)
$HgCl_2$ (0.05%)	2	6.67(14.96)	16.67(24.01)	11.67(19.48)
$HgCl_2$ (0.05%)	4	16.33(23.82)	19.34(26.07)	17.84(24.94)
$HgCl_2$ (0.05%)	6	22.67(28.41)	19.33(26.07)	21.00(27.24)
$HgCl_2$ (0.1%)	2	34.66(36.05)	44.67(41.92)	39.67(38.98)
$HgCl_2$ (0.1%)	4	60.66(51.14)	67.33(55.12)	64.00(53.13)
$HgCl_2$ (0.1%)	6	31.66(34.22)	45.00(42.11)	38.33(38.16)
$HgCl_2$ (0.2%)	2	13.00(21.12)	26.00(30.60)	19.50(25.86)
$HgCl_2$ (0.2%)	4	16.00(23.39)	19.34(26.07)	17.67(24.73)
$HgCl_2$ (0.2%)	6	13.33(21.32)	20.33(26.73)	16.83(24.02)
Mean		21.50(25.44)	27.80(29.86)	
$CD_{(0.05)}$	Explant (E) = 0.85 Sterilant (S) = 1.90 E × S = 2.69			

*Arcsine transformed values in parenthesis

Table 5. Effect of sodium hypochlorite (NaOCl) on per cent survival cultures in apple clonal rootstocks during April of 2021

Treatment	Duration	MM106		
		Shoot tip	Nodal segment	Mean
Control	-	0.00(0.00)*	0.00(0.00)	0.00(0.00)
NaOCl (3%)	10	6.67(14.96)	13.00(21.12)	9.83(18.04)
NaOCl (3%)	20	9.33(17.76)	16.67(24.01)	13.00(20.92)
NaOCl (3%)	30	18.67(25.57)	26.67(31.05)	22.67(28.31)
NaOCl (8%)	10	32.66(34.84)	41.67(40.18)	37.17(37.51)
NaOCl (8%)	20	49.44(44.66)	60.00(50.75)	54.72(47.71)
NaOCl (8%)	30	35.00(36.22)	44.96(42.09)	39.98(39.15)
NaOCl (12%)	10	16.67(24.01)	27.00(31.27)	21.83(27.64)
NaOCl (12%)	20	16.00(23.39)	20.00(26.55)	18.00(24.97)
NaOCl (12%)	30	13.33(21.40)	16.67(24.01)	15.00(22.71)
Mean		19.78(24.28)	26.66(29.13)	
$CD_{(0.05)}$	Explant (E) = 0.97 Sterilant(S) = 2.17 E × S = 3.07			

*Arcsine transformed values in parenthesis

0.1% $HgCl_2$ for 4 minutes. The findings were very beneficial for mass clonal apple rootstock multiplication in a local setting, and they may provide suggestions for establishing commercial units for the propagation of such clonal apple rootstock types.

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Conflict of interest

The authors declare that they have no conflict of interest.

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