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In vitro multiplication of peach rootstocks

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ABSTRACT

The leaf yellowing and senescence during micro-propagation reduces the shoot proliferation rates with every sub-culture in peach rootstock (*Prunus* spp.). The effect of culture media and supplements on the proliferation of cultures during the micro-propagation of two peach rootstocks 'Sharbati' and 'Flordaguard' was studied. In both *Prunus* genotypes, the proliferated cultures decreased from 77.3% and 67.0 % after the first sub-culture to 35.3 % and 27.3 % after the third sub-culture in 'Sharbati' and 'Flordaguard', respectively. QL medium significantly improved the proportion of proliferated cultures over MS, WPM, DKW and the modified MS media. The highest proliferated cultures (79.0% and 70.7%) and shoot number per culture (4.2 and 3.7) were recorded after the third subculture, in 'Sharbati' and 'Flordaguard', respectively with QL medium. Both the rootstocks varied in their response to iron chelates. The higher proliferation rates were obtained with MS medium by substituting Fe-EDTA (0.1 mM) with Fe-EDDHA (0.1-0.3 mM) in 'Sharbati' and with Fe-Na EDTA (0.1 mM) in 'Flordaguard'. In 'Sharbati', the highest proliferated cultures (44.3%) after the third subculture were observed with Fe-EDDHA (0.3 mM). In 'Flordaguard', the highest proportion of proliferated cultures (45%) after the third subculture was recorded with Fe-Na EDTA (0.1 mM). Adding silver nitrate (3 mM) improved the shoot proliferation rates to the highest levels (63% and 51%) after third subculture in 'Sharbati' and 'Flordaguard', respectively. The highest rooting (34.3% and 38.0%) and number of roots per shoot (2.2 and 2.5) were recorded with 3.75 mM of IBA in 'Sharbati' and 'Flordaguard', respectively.

Key words: *Prunus* spp., Fe- EDDHA, Silver nitrate, QL media

INTRODUCTION

In the sub-tropical regions of India, seedlings of peach cv. 'Sharbati' are used as a rootstock but are highly susceptible to root-knot nematode. Peach rootstock 'Flordaguard' is promising regarding fruit yield, quality, and resistance against the root-knot nematode. Therefore, traditional propagation methods like seedlings and cuttings must be refined or replaced with modern techniques (micro-propagation) to enable the mass multiplication of peach rootstocks. Efficient *in vitro* clonal propagation protocols have been developed for different rootstocks. However, it has been observed that many shoots generally degenerate due to yellowing and hyperhydricity during the micropropagation of stone fruits. Micro-propagation of peach is a little more complex than the other fruit crops of the genus *Prunus* (Adelberg *et al.,*1). During the commercial micropropagation of peach rootstocks in our laboratory, cultures were lost due to yellowing of leaves, leaf senescence and subsequent drying of affected cultures (Plate 1). This incidence of leaf curling, yellowing, and drying of cultures increases with sub-culturing, reducing the shoot proliferation rates of *in vitro* cultures. Similarly, the problem also reduces the multiplication rates in cherry (Alanagh *et al.,* 2).

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MS medium, the most used medium for micro-propagation of fruit trees (Kaur *et al*., 7), has not been found suitable for regeneration of apples and pears (Nowak *et al*., 11). The higher levels of ammonium versus nitrate ions negatively affected the regeneration of plum cultures (Nowak *et al.,* 11). In cherries, the QL, DKW, and WPM media *have been reported as most suitable for* bud regeneration from leaf explants (Matt and Jehle, 10). However, Gerdakaneh **(**6) obtained the highest shoot proliferation in *Prunus* rootstock 'GF-677' cultures with MS medium over B5 and WPM. Antonopoulou *et al.* (3) obtained better rooting of peach × almond rootstock 'GF-677' by substituting Fe-EDTA with iron salt of ethylene diamine di-2-hydroxyphenyl acetate (Fe-EDDHA; 6% Fe). Replacement of Fe-EDTA with Fe-EDDHA in the DKW-C medium improved the shoot growth, rooting, root growth and diminished leaf chlorosis in walnuts (Licea-Moreno *et al*., 9). Adding silver nitrate in the medium improves shoot growth and leaf expansion in anthurium (Cardos, 4). *In vitro* yellowing and defoliation of shoots and death of cultures were overcome with silver nitrate in the micro-propagation of moringa (Drisya Ravi *et al.,* 5).

It was hypothesised that different culture media and supplements like iron salts, calcium, iron and silver nitrate could influence the shoot multiplication rates during peach micro-propagation. The present

Plate 1. Yellowing and senescence in *in vitro* cultures of peach.

investigations were conducted to study the effect of different supplements and media compositions on *in vitro* propagation of two *Prunus* rootstocks *viz.*, 'Sharbati' and 'Flordaguard'.

MATERIALS AND METHODS

The present studies were made during 2018-2021. The explants were collected from a single plant of two *Prunus* rootstocks included in the studies *viz*., 'Sharbati' and 'Flordaguard' growing at the Fruit Research Farm, PAU Ludhiana. The nodal segment explants (1.5 cm) were surface sterilized with mercuric chloride (0.1%) for three min and cultured on a medium fortified with benzyl adenine (4.44 mM). The peach genotypes were cultured in test tubes on seven tissue culture media (Table 1) *viz*., Murashige and Skoog (MS) medium, Woody Plant Medium (WPM), Quoirin and Lepoivre (QL) medium, Driver and Kuniyuki (DKW) medium and three modified MS media (MMS1, MMS2 and MMS3). All seven media were fortified with benzyl adenine (4.44 mM) during the explant's establishment. After four weeks, the established explants were proliferated on the same media with BA (4.44 mM) and Kinetin (2.32 mM) in 250 ml jam jar bottles with 50 ml media. The proliferated shoots were sub-cultured after every 6 weeks.

Besides, to study the effect of various chemicals on the loss of cultures due to leaf yellowing and senescence, the peach rootstocks were also proliferated on MS media fortified with BA (4.44 mM) + Kinetin (2.32 mM) and different levels of EDTA, $FeCl_3$, Fe-EDDHA, Fe-EDTA, Fe-NaEDTA, silver nitrate, calcium chloride, boric acid and magnesium sulphate (Fig. 1 - 2). Data regarding the percentage of cultures showing shoot proliferation and the number of shoots per culture were recorded every six weeks for each sub-culture. The *in vitro* raised shoots (>1.5 cm) obtained after shoot multiplication were cultured on MS medium fortified with IBA (0.25-2.0 mg/l). The data regarding per cent root induction, number of roots per shoot and average root length were recorded after four weeks of transfer on rooting media.

The data were subjected to analysis of variance (ANOVA) using statistical software SAS 9.3. The mean separation was done using the least significant difference (Fisher's LSD) at $P \leq 0.05$ following significant *F* test.

RESULTS AND DISCUSSION

Developing efficient micro-propagation protocols for fruit crops like *Prunus* sp. is still challenging for *in vitro* plant culture researchers (Alanagh *et al.,* 2). The highest shoot proliferations (79.9 and 74.6 in 'Sharbati' and 'Flordaguard', respectively) were recorded after the first sub-culture (Table 2 - 3). After the second sub-culture, irrespective of the culture media, 58.8% and 49.6% shoot proliferations were recorded in 'Sharbati' and 'Flordaguard', respectively. Further, the shoot proliferation decreased to 43.2% and 36.0% in 'Sharbati' and 'Flordaguard', respectively, after the third sub-culture. Similarly, there was a significant decrease in the number of shoots per culture from the first sub-culture (4.6 and 4.4 shoots per culture) to the third sub-culture (2.4 and 1.8 shoots per culture) in the respective cultivars. The shoot necrosis and vitrification resulted in lower survival rates of *Prunus* rootstock cultures with MS medium. The poor results with MS media

Table 1. Composition (mg/L) of media used for shoot proliferation.

Media	Ammonium nitrate	Potassium nitrate	Potassium sulphate	Calcium nitrate	Calcium chloride	Magnesium sulphate	Potassium dihydrogen phosphtae	Sodium dihydrogen phosphate	Manganese sulphate	Ferrous sulphate
MS	1650	1900	0	0	440	370	170	0	22.3	27.8
WPM	400	0	990	556	96	370	170	0	22.3	27.8
QL	400	1800	0	599	0	176	270	0	0.76	27.8
DKW	1416	0	1559	1368	149	361	265	0	33.5	33.8
MMS1	800	25	0	0	440	540	300	50	22.3	27.8
MMS2	1650	1900	0	800	0	370	170	0	22.3	27.8
MMS3	800	25	0	400	0	370	300	50	22.3	27.8

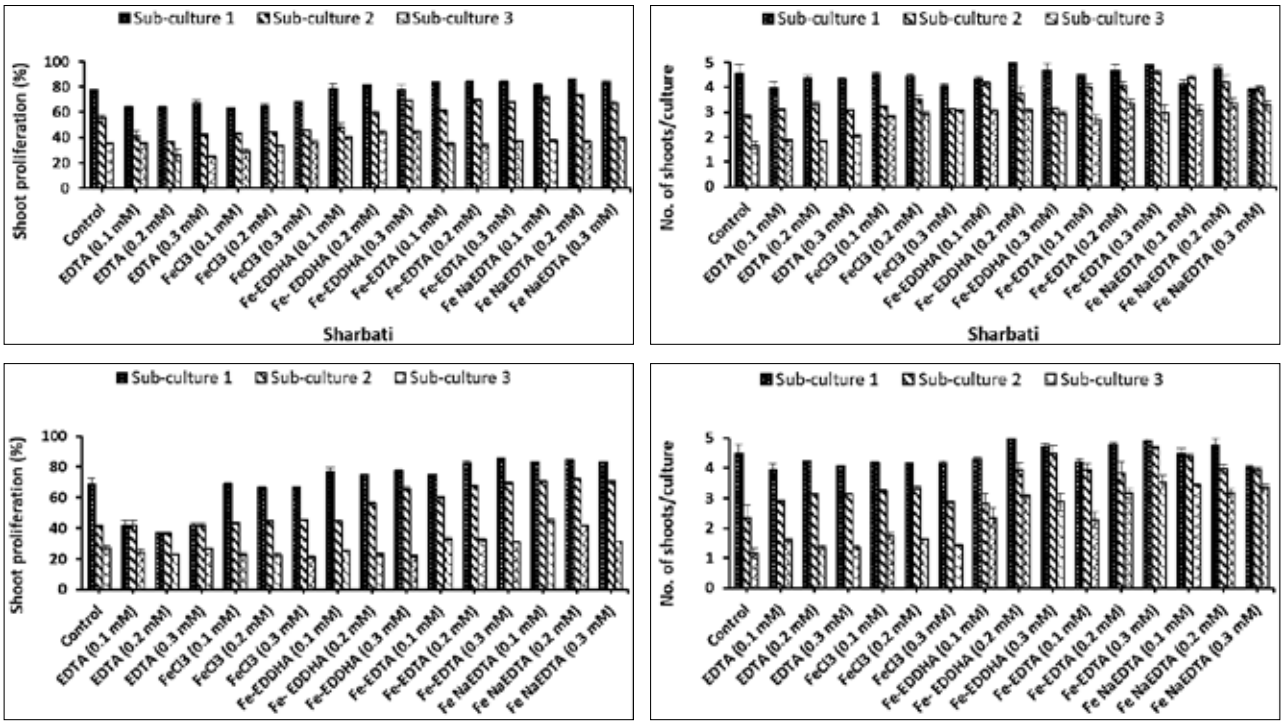

Fig. 1. Effect of EDTA, $FeCl_3$, Fe-EDDHA, Fe-EDTA, Fe-NaEDTA on shoot proliferation.

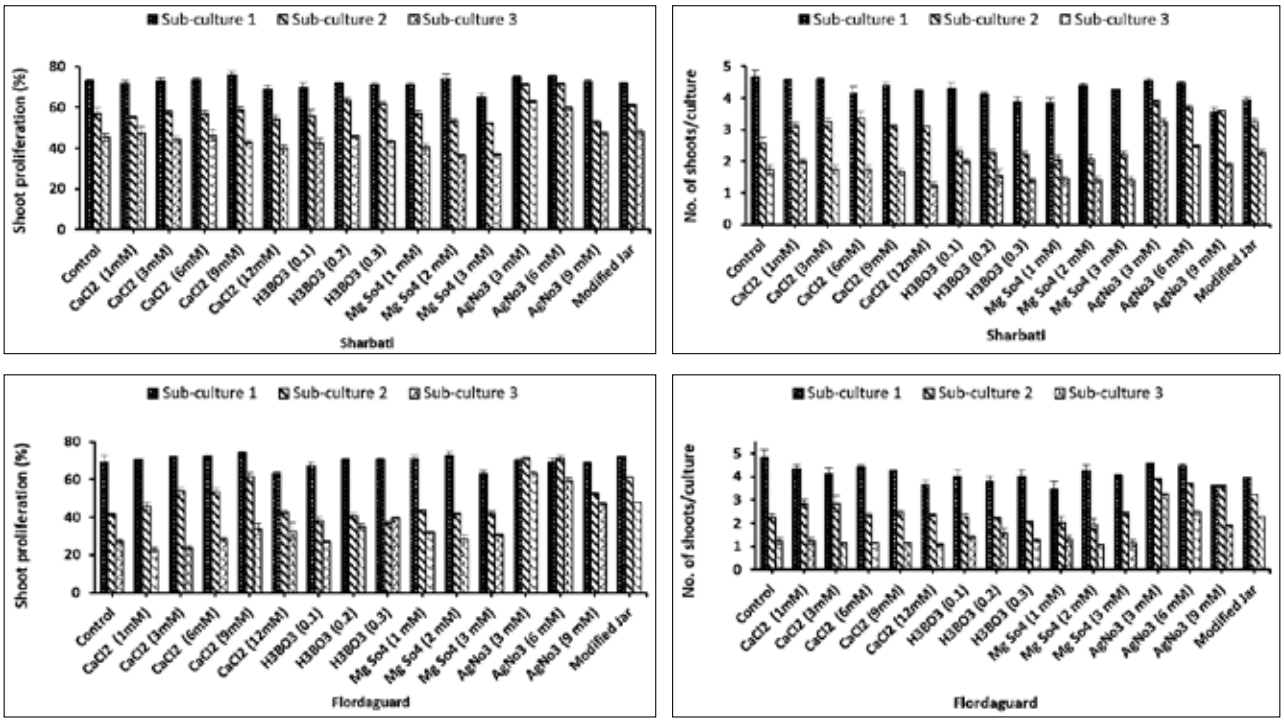

Fig. 2. Effect of silver nitrate, calcium chloride, boric acid, and magnesium sulphate and aerated vessel on shoot proliferation.

Table 2. Effect of media compositions on shoot proliferation in 'Sharbati'.

	Proliferated cultures (%)				Shoot number/ culture			
	Sub-culture 1	Sub-culture 2	Sub-culture 3	Mean	Sub-culture 1	Sub-culture 2	Sub-culture 3	Mean
MS	78.3±1.5^{cd}	53.0±2.5^{h}	38.0±1.7^{kl}	56.4^{c}	4.7±0.12^{ab}	2.6±0.12^{c}	1.6±0.06^{d}	3.0^{e}
WPM	83.7±1.5^{b}	56.3±0.9^{gh}	40.7±1.5^{jk}	60.2^{b}	4.8±0.24^{ab}	3.6±0.15^{b}	2.0±0.12^{c}	3.5^{d}
QL	88.3±1.9^{a}	79.7±1.5^{bcd}	79.0±0.6^{cd}	82.3^{a}	5.0±0.12^{a}	4.4±0.06^{a}	4.2±0.19^{a}	4.5^{a}
DKW	71.0±0.6^{e}	47.7±1.3^{i}	34.3±1.2^{lm}	51.0^{d}	3.4±0.15^{c}	2.6±0.15^{c}	1.4±0.12^{d}	2.4^{f}
MMS1	79.3±0.9^{cd}	57.7±3.0^{fg}	35.7±1.2^{lm}	57.6^{c}	4.7±0.12^{ab}	4.0±0.09^{ab}	2.0±0.06^{c}	3.6^{cd}
MMS2	77.0±1.5^{d}	56.7±1.5^{fgh}	31.3±1.8^{m}	55.0^{c}	4.8±0.06^{ab}	4.4±0.12^{a}	2.1±0.09^{c}	3.7^{bc}
MMS3	81.7±1.8^{bc}	61.0^{f}	43.3±0.9^{ij}	62.0^{b}	4.4±0.12^{b}	3.6±0.39^{b}	3.6±0.09^{b}	3.9^{b}
Mean	79.91^{a}	58.8^{b}	43.2^{c}		4.6^{a}	3.6^{b}	2.4^{c}	
LSD (0.05)	Media:2.63 Sub-culture:1.72 Media × Sub-culture: 4.56				Media:0.25 Sub-culture:0.16 Media ×- Sub-culture: 0.43			

The values followed by same superscript in a column did not differ significantly ($p \leq 0.05$); ± S.E.

Table 3. Effect of media compositions on shoot proliferation in 'Flordaguard'.

	Proliferated cultures (%)				Shoot number/ culture			
	Sub-culture 1	Sub-culture 2	Sub-culture 3	Mean	Sub-culture 1	Sub-culture 2	Sub-culture 3	Mean
MS	69.0±3.8^{d}	41.3±0.9^{gh}	26.7±2.9^{jk}	45.7^{d}	4.5±0.20^{ab}	2.3±0.29^{c}	1.2±0.09^{c}	2.7^{d}
WPM	78.0±2.1^{ab}	52.3±2.9^{e}	31.7±3.2^{ij}	54.0^{b}	4.4±0.22^{ab}	2.9±0.06^{b}	1.7±0.12^{b}	3.0^{b}
QL	75.3±2.0^{bc}	72.3±2.0^{cd}	70.7±1.9^{cd}	72.8^{a}	4.6±0.12^{a}	4.1±0.23^{a}	3.7±0.12^{a}	4.1^{a}
DKW	69. ±0.9^{d}	37.7±0.9^{h}	26.0±1.2^{k}	44.3^{d}	4.0±0.12^{c}	2.7±0.06^{bc}	1.8±0.06^{b}	2.8^{cd}
MMS1	74.0±1.0^{bcd}	49.3±1.0^{ef}	26.0±0.6^{k}	49.8^{c}	4.2±0.03^{bc}	2.9±0.22^{b}	1.3±0.09^{c}	2.8^{cd}
MMS2	75.3±1.5^{bc}	47.7±1.5^{ef}	26.0±1.5^{k}	49.8^{d}	4.2±0.03^{bc}	2.6±0.03^{bc}	1.2±0.09^{c}	2.7^{d}
MMS3	81.0±1.2^{a}	46.7±1.2^{fg}	36.0±0.6^{hi}	54.6^{b}	4.6±0.09^{a}	2.9±0.09^{b}	1.7±0.09^{b}	3.1^{b}
Mean	74.6^{a}	49.6^{b}	34.7^{c}		4.4^{a}	2.9^{b}	1.8^{c}	
LSD (0.05)	Media:3.19 Sub-culture:2.09 Media × Sub-culture: 5.5				Media:0.22 Sub-culture:0.14 Media × Sub-culture: 0.38			

The values followed by same superscript in a column did not differ significantly ($p \leq 0.05$); ± S.E.

might be due to higher nitrogen levels in the MS medium (Perez-Tornero *et al.,* 12). Higher ammonium ions in the MS medium have been reported to be a reason for poor survival of cultures and higher shoot necrosis and hyperhydricity in *Prunus sp.* (Alanagh *et al.,* 2). Higher ammonium to nitrate ion ratio in MS medium has also been reported to hamper plant regeneration (Nowak *et al.,* 11). The lower nutrient levels in the WPM make this medium suitable for micropropagation of woody plants. The highest proliferated cultures and shoot number per culture in both 'cultivars were recorded on QL media. The QL media improved the proportion of live proliferated cultures and shoot number per culture in 'Sharbati' and 'Flordaguard' after every subculture (Table 2 - 3). After the third subculture, QL medium increased the proportion of live proliferated cultures by 108 and 165% over the MS media in 'Sharbati' and 'Flordaguard'. This was followed by MMS3 and WPM media, which increased the proportion of proliferated cultures after the third subculture in 'Sharbati' (7 and 14%, respectively) and 'Flordaguard' (19 and 35%, respectively). The QL medium (Table 1) had medium or rather optimum levels of macronutrients with low ammonium and high calcium levels without chloride ions (Matt and Jehle, 10**).** The WPM and QL media have a lower ammonium to nitrate ion ratio over MS medium, making it suitable for woody plants, including the *Prunus* sp. (Perez-Tornero *et al.,* 12).

In MS medium, iron sulphate (0.1M) is added along with disodium EDTA (0.1M). Hence, iron is present as Fe-EDTA. However, while micro-

propagating the two peach rootstocks, the defoliation problems, poor growth and yellowing of cultures were observed during the shoot multiplication, which increased at every subculture. After the first subculture, 77.3% and 67.0 % of cultures survived in 'Sharbati' and 'Flordaguard', respectively (Fig. 1). The number of surviving cultures decreased after the second subculture (55.3 and 39.7 per cent, respectively, in 'Sharbati' and 'Flordaguard') and the third subculture (35.3 and 27.3 per cent in 'Sharbati' and 'Flordaguard', respectively). Increasing the EDTA levels alone had a detrimental effect on the survival of cultures. EDTA undergoes photooxidation and forms formaldehyde, which can be toxic to plants. Due to the oxidation of EDTA, iron from unavailable precipitates and becomes unavailable. Replacement of the Fe-EDTA (0.1 M) in the MS medium with Fe-NaEDTA (0.1 - 0.3M) or addition of additional Fe-EDTA (0.1 - 0.3M) improved the proportion of live cultures after the first and second subculture in both 'Sharbati' and 'Flordaguard' rootstocks (Fig. 1). Fortification of MS medium with Fe-EDDHA, Fe-EDTA, Fe-NaEDTA and ferric chloride has a significant effect on the proportion of proliferated cultures at each subculture. In Sharbati and Flordaguard rootstocks, Fe-NaEDTA and Fe-EDTA improved the proportion of live cultures after the first and second subcultures. However, Fe-EDDHA resulted in the highest culture survival in 'Sharbati' and Fe-NaEDTA in 'Flordaguard' after the third subculture. Fortification of MS media with ferric chloride had a detrimental effect on the survival of cultures in every subculture compared to MS media. Fe-NaEDTA (0.2 mM) resulted in the highest proportion of proliferated cultures after the first (85.7% and 84.0%) and second (73.7 and 72%) subcultures in the respective rootstocks, respectively. The percentage of proliferated cultures with Fe-NaEDTA (0.2 mM) did not differ from the proliferated cultures with Fe-NaEDTA (0.1 mM), Fe-EDTA (0.2 mM) and Fe-EDDHA (0.3 mM). After the third subculture, the highest proportion of proliferated cultures (44.3%) in 'Sharbati' was recorded with Fe-EDDHA (0.3 mM), which did not differ from the proliferated cultures in Fe-EDDHA (0.1 and 0.2 mM). In 'Flordaguard', the highest proportion of proliferated cultures (45%) after the third subculture was recorded with Fe-NaEDTA (0.1 mM), which was closely followed by proliferated cultures Fe-NaEDTA (0.2 mM). The number of shoots per culture decreased with every subculture. The highest number of shoots per culture was recorded after the first subculture and the minimum after the third subculture. Fe-NaEDTA (0.1 and 0.2 mM) and Fe-EDTA (0.2 and 0.3 mM) resulted in higher shoot numbers in both rootstocks. Fe-NaEDTA (0.2 mM) resulted in the highest shoot number in 'Sharbati' (5.2 cm) and 'Flordaguard' (5.0 cm) after the first subculture. After the second subculture, the shoot number in both the rootstocks was lesser with Fe-EDDHA than with Fe-NaEDTA and Fe-EDTA. However, after the third subculture, the shoot number in sodium ferric EDDHA (0.2 mM) was similar to the Fe-NaEDTA (0.1 and 0.2 mM) and Fe- EDTA (0.2 and 0.3 mM). Though improvement in the survival of cultures with the substitution of Fe-EDTA with Fe-EDDHA has been reported in walnuts, the reasons for the superiority of Fe-EDDHA were uncertain (Licea-Moreno *et al.,* 9).

After the first subcultures, in both 'Sharbati' (76%) and 'Flordaguard' (74%) rootstocks, the highest shoot proliferation (Fig. 2, Plate- 2) was recorded with the fortification of MS medium with calcium chloride (9 mM). However, after the second and third subcultures, the highest shoot proliferation rates and shoot number were recorded with silver nitrate (3 mM). However, the addition of calcium chloride, boric acid, magnesium sulphate and modified jar failed to improve the shoot proliferation over the MS media after the second and third subcultures in both the genotypes. The addition of silver nitrate (0.3 mM) improved the survival of the cultures by 87 and 145% over the MS media in 'Sharbati' and 'Flordauard', respectively (Fig. 2). In the *in vitro* cultures, ethylene synthesis led to physiological disorders like excessive callusing, leaf abscission, and yellowing of leaves (Yasmin *et al.,*14), which reduces the micropropagation rates. Silver ions block ethylene receptors and improve *in vitro* axillary shoot proliferation and root formation (Lai *et al.,* 8). The addition of silver nitrate in the culture medium could not prevent ethylene accumulation but prevented its adverse effects (Cardos, 4). Silver overcomes cultures' abscission, senescence and growth inhibition during micropropagation (Drisya Ravi *et al.,* 5). The modified culture vessel also improved the survival of cultures by 31% and 77% over the MS medium. This might be a result of better aeration, lower humidity and ethylene levels in the

Plate 2. (A) Shoot proliferation on MS medium + BA (4.44 mM), Kinetin (2.32 mM) and $AgNO_3$ (6 mM) (B) Rooting with IBA (3.75 mM) (C) Hardening of peach rootstock.

Table 4. Effect of IBA levels on rooting percentage, root number and root length.

IBA level (mM)	Sharbati			Flordaguard		
	Rooting percentage (%)	Number of roots	Root length (cm)	Rooting percentage (%)	Number of roots	Root length (cm)
QL medium	0.0±0^{d}	0.0±0^{c}	0.0±0^{e}	0.0±0^{d}	0.0±0^{c}	0.0±0^{e}
QL + IBA (1.25)	13.7±0.88^{c}	1.3±0.17^{b}	2.4±0.03^{b}	16.3±0.80^{c}	1.8±0.15^{b}	4.5±0.12^{b}
QL + IBA (2.5)	24.7±1.45^{b}	1.9±0.07^{a}	2.6±0.10^{a}	34.2±2.05^{ab}	2.3±0.20^{a}	4.3±0.02^{b}
QL + IBA (3.75)	34.3±2.34^{a}	2.2±0.17^{a}	1.7±0.07^{c}	38.0±2.08^{a}	2.5±0.18^{a}	4.8±0.06^{a}
QL + IBA (5.0)	26.0±2.08^{b}	1.5±0.03^{b}	1.1±0.03^{d}	32.3±1.45^{b}	1.6±0.15^{b}	2.1±0.10^{c}
QL + IBA (10.0)	0.0±0.0^{d}	0.0±0^{c}	0.0±0^{e}	2.7±0.88^{d}	1.4±0.06^{b}	1.4±0.09^{d}
LSD (0.05)	4.5	0.3	0.2	4.4	0.44	0.22

The values followed by same superscript in a column did not differ significantly ($p \leq 0.05$); ± S.E.

modified jars (Fig. 2). The accumulation of ethylene in non-ventilated vessels caused leaf yellowing and leaf abscission in pepper cultures (Santana-Buzzy *et al.,*13). The modified jar followed the silver nitrate (3 mM) in improving the number of shoots per culture over the basal MS Medium (control) in 'Sharbati' and 'Flordaguard' cultures during all three sub-cultures. After the second and third sub-cultures, except for the silver nitrate, no other adjuvants, viz. magnesium sulphate, calcium chloride, and boric acid, could improve the shoot proliferation and shoot number per culture in both the genotypes.

The highest rooting percentage (34.3 and 38.0%) and number of roots per shoot (2.2 and 2.5) were recorded with indole-3-butyric acid (IBA) 3.75 mM in both the genotypes 'Sharbati' and 'Flordaguard', respectively (Table 4). The highest root length of 2.2 cm was recorded with an IBA of 1.25 mM in 'Sharbati' and 4.8 cm with an IBA of 3.75 mM in' Flordaguard'.

AUTHORS' CONTRIBUTION

Conceptualization, design, methodology: AT and GSS; Investigation: AT, GSS, HS; Statistical analyses: AT and GSS; Manuscript writing: AT, GSS, HS.

DECLARATION

The authors declare no conflict of interest.

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