An efficient and reliable protocol on in vitro propagation of ‘Colt’ (Prunus avium F 299/2 x Prunus pseudocerasus Lind) rootstock of cherry

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Abstract

Background The 'Colt' is a triploid hybrid of P. *avium* and P. *pseudocerasus*L. grown as a cherry rootstock because of its resistance to cherry stem pitting (a debilitating virus disease), Phytophthora root rot, bacterial canker, and gopher damage.

ResultsThe current study was carried out using two types of explants: forced (F_1) and unforced (F_2) and five types of sterilants were used to sterilize explants: 10% sodium hypochlorite (NaOCl) for 10 minutes (S1), 0.1% mercuric chloride (HgCl2) for 5 minutes (S2), ethyl alcohol for 10 seconds (S3), (S1) + (S3) & (S2) + (S3) for sterilization. During culture establishment, six growth regulator regimes *viz.,* BA (Benzyl adenine) (0.20), (0.40) & (0.60) and BA+IBA (Indole-3-butyric acid) (0.20+0.01), (0.40+0.01), and (0.60+0.01) mg/l, two media i.e., MS (Murashige and Skoog, 1962)& WPM (Woody Plant Medium)& two types of explants viz., forced (F_1) & unforced shoot tips(F_2) were utilized. During shoot proliferation, MS & WPM culture medium with growth regulators viz., BA (0.20), (0.40) & (0.60), BA+IBA (0.20+0.01), (0.40+0.01) and (0.60+0.01) mg/l were used respectively. During rhizogenesis using different media (MS & WPM) with various levels of IBA (0.50), (1.00) (1.50), and (2.00) mg/l, respectively were used. The superior rooted plants were hardened in sand & cocopeat respectively.

Conclusion In this experiment, maximum culture asepsis (%) obtained in unforced shoot tips(F_2) with (S_5),maximum explant survival (%) in forced shoot tips (F_1) with (S_4), and highest establishment (%) with MS medium, BA (0.6) mg/l & in forced shoot tips (F_1). Significantly higher shoot proliferation is obtained with MS medium with BA (0.6) mg/l , maximum shoot number & length in WPM medium with BA (0.2) mg/l. A significantly higher rooting percentage was obtained in MS medium with IBA (1.00) mg/l with maximum *ex vitro* survival (%) in cocopeat.

Background

Cherry (*Prunus avium*): small stone fruits belonging to family rosaceae are high in K, Ca, Vitamins(A and C), Na, protein, carbohydrates, Mg, Fe, and fat, as well as antioxidants and anti-inflammatory compounds [1,2]. Cherries are thought to have originated In Asia Minor, between the Black Sea and the Caspian Sea [3]. Turkey was the world's largest producer of cherries, producing approximately 874 thousand metric tons [4]. Jammu and Kashmir is the leading producer of cherry in India, with a 2700-hectare area and a production of 15555 metric tonnes [5]. Cherry is the most abundantly grown temperate fruit in the world. Cherry trees, like the majority of fruit crops, are propagated by grafting the scion wood onto rootstock. Seedling rootstocks are not uniform, and tree vigour and bearing age vary greatly. Furthermore, in recent years, the average land holding of orchardists has decreased, resulting in the replacement of traditional cherry orchards with standard trees with wider spacing with high-density planting systems with dwarfing clonal rootstocks [6]. The dwarf rootstocks of Sweet cherry are nowadays used in the production of intensive orchards [7]. When compared to standard rootstocks, dwarf and semi-dwarf rootstocks are particularly helpful in increasing fruit quality and efficiency [8]. In today's intensified fruit production conditions, there is a high demand for dwarf rootstocks enabling restricted plant growth resulting in trees with a compact crown, thus promoting easy harvesting and maintenance [9]. Aside from the high productivity of grafted varieties, the advantages of modern clonal rootstocks include genetic homogeneity, compatibility with varieties, good fixation in soil and almost complete absence of root growth, resistance to changing environmental abiotic and biotic stress and ease of vegetative propagation [10]. The rootstock is an important part of any orchard management system. Dwarfing cherry rootstocks and newer cultivars have opened up new opportunities for developing high-density sweet cherry orchards. Colt' (*Prunusavium* F 299/2 x *Prunus pseudocerasus* Lind) cherry rootstock belonging to family Rosaceae originated in Kent, England, by H.M. Tyderman, late of East Malling, England. The East Malling Research Station in Maidstone, Kent, England, was used for pollination, evaluation, and testing[11]. The 'Colt' cherry is a triploid hybrid of P. *avium* and P. *pseudocerasus*L. grown as a cherry rootstock [12].

Because of its resistance to cherry stem pitting, a debilitating virus disease, 'Colt' has been widely planted. It is resistant to Phytophthora root rot, bacterial canker, and gopher damage, but susceptible to crown gall. 'Colt' performs well in replant situations where cherries follow cherries on non-fumigated sites in the Pacific Northwest (PNW) [13].

Without the use of healthy planting material,fruit crop cultivationis currently impossible. Clonal micropropagation is a popular approach for commercially producing fruit stocks [4, 14]. Growing stone fruit crops on clonal rootstocks makes it easier to

obtain sterile, virus-free planting stock [9]. The advantages of clonal micropropagation of plants are relatively short reproductivephase and a higher yield of marketable produce [15].

The clonal planting stock is generally commercially propagated using traditional methods such as mound layering. These methods face some drawbacks likecomplete season dependence, lower multiplication ratio, need of lot of space and labour, and yield unprofitable planting material. In vitro propagation techniques, on the other hand, provide an effective method of producing high-quality true to type planting material in less time and space [16].

Over the last two decades, many researchers have used micro-propagation under suitable in-vitro conditions to produce healthy, true-to-type, disease-resistant plants and with desirable traits in cherry scions and rootstocks. Different genotypes require different systems of micropropagation, such as explant type, culture establishment, and sterilization techniques, rooting media, and hardening procedures. Furthermore, the levels and types of plant growth regulators differ and exhibit different responses with different explant types [17, 14,18, 6, 19, 20].The aim of the present study was to develop a reliable and efficient protocol for fast *in vitro* propagation of cherry rootstock “Colt” to boost/increase production.

Results

Culture asepsis and explant establishment

For sterilization of explants five type of sterilants have been used in this experiment *viz.*,10% sodium hypochlorite (NaOCl) for 10 minutes (S_1), $HgCl_2$ for 5 minutes at 0.1% concentration(S_2), Ethyl alcohol for 10 second duration (S_3),10% sodium hypochlorite (NaOCl) for 10 minutes followed by Ethyl alcohol for 10 second duration (S_4) &$HgCl_2$ (0.1%) for 5 minutes followed by Ethyl alcohol for 10 seconds (S_5) with two types of explants viz., forced (F_1) & unforced shoot tips(F_2). After about four weeks of sterilization, culture asepsis (%) & explant survival (%) were estimated with maximum culture asepsis (%) obtained in unforced shoot tips(F_2) with 0.1% $HgCl_2$ for 5 minutes + Ethyl alcohol for 10 seconds (S_5) (60.66%) followed by forced shoot tips(F_1) with10% sodium hypochlorite (NaOCl) for 10 minutes + Ethyl alcohol for 10 seconds (S_4) (60.66%)(55.80%) as shown in Table (1). In this experiment, maximum explant survival (%) (60.33%) was obtained in forced shoot tips (F_1) with 10% sodium hypochlorite (NaOCl) for 10 minutes followed by Ethyl alcohol for 10 seconds (S_4) followed by unforced shoot tips (F_2) with 10% sodium hypochlorite (NaOCl) for 10 minutes (S_1) (50.66%) as shown in (Table 2).

Six growth regulator regimes *viz.*, BA (Benzyl adenine) (0.20), (0.40) & (0.60) and BA + IBA (Indole-3-butyric acid) (0.20 + 0.01), (0.40 + 0.01), and (0.60 + 0.01) mg/l, two media i.e., MS & WPM & two types of explants viz., forced (F_1) & unforced shoot tips(F_2) were utilized to standardize explant establishment in “Colt” rootstock of cherry. In this study as shown in (Table 3) highest establishment percentage (83.33%) with MS medium, BA (0.6) mg/l & in forced shoot tips (F_1) followed by WPM medium, BA (0.2 & 0.4) mg/l and forced shoot tips (F_1) with (81.00%) depicted in (Fig. 1a).

Shoot proliferation

Since for subculture, the sterile forced shoot tip cultures that established successfully on the primary culture medium were cut into 0.5–1.0 cm long stem segments.These micro-shoots were then inoculated inMS & WPM supplemented with growth regulators viz., BA (0.20), (0.40) & (0.60), BA + IBA (0.20 + 0.01), (0.40 + 0.01) and (0.60 + 0.01) mg/l respectively for shoot proliferation. Significantly higher shoot proliferation (92.00%) is obtained with MS medium with BA (0.6) mg/l (Table 4) followed by WPM medium with BA (0.2) mg/l (87.00%), maximum shoot number (5.83) in WPM medium with BA (0.2) mg/l followed by MS medium, BA (0.4) mg/l (Table 5) & maximum shoot length (16.16 mm) in WPM medium with BA (0.2) mg/l followed by WPM medium with BA + IBA (0.20 + 0.01) mg/l with (14.16mm)(Table 6) (Fig. 1b).

Rhizogenesis and Hardening

The rooting characteristics of cherry rootstock “Colt” (rooting %,root number & root length (mm)) were observed after the sterile proliferated shoot tip cultures were subjected to rhizogenesis using different media (MS & WPM) with various levels of IBA (0.50), (1.00) (1.50), and (2.00) mg/l, respectively. Significantly higher rooting percentage (90.66%) wasattained in MS medium

with IBA (1.00) mg L^{-1} followed by 2.00 mg/l IBA (85.66%) (Table 7). Highest root number (5.92) was achieved with 1.00 mg/l IBA followed by 2.00 mg/l IBA (4.75) (Table 8). Also, maximum root length (70.16 mm) was achievedwith 1.00 mg/l IBA followed by 2.00 mg/l IBA (61.63) (Table 9) (Fig. 1c).

The plantlets with maximum rooting rate (%) were carefully removed from the culture vessels and cleaned thoroughly under running tap water to remove any residual agar adhering to roots. Those plantlets were then placed in covered jars containing cocopeat & sand (100%) respectively. Observations on the survival percentage of rooted plantlets were recorded after 4 weeks. In this experiment, it was found that maximum *ex-vitro* survival (%) was observed in plantlets hardened with (74.97%) success in cocopeat followed by sand(58.33%) (Table-10) (Fig. 1d)

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Table 1
Influence of sterilants and explant origin on culture asepsis (%) of cherryrootstock "Colt"(*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind)

Explant origin (F)	**Sterilants (S)** S_1	S_2	S_3	S_4	S_5
F_1	16.16	13.13	52.60	55.80	41.16
F_2	45.83	33.16	23.50	22.53	**60.66**
C.D($P \leq 0.05$)			**Explant (F)**	0.48	
			Sterilants (S)	0.76	
			(F)X (S)	1.08	

The data was analysed by comparing means using one-way ANOVA, and the significance was determined by CD $_{(p \leq 0.05)}$ using OPSTAT(v.6.8).Forced shoot tips(F_1) and Unforced shoot tips(F_1) are the two explants types and 10% sodium hypochlorite (NaClO) for 10 minutes (S_1), 0.1% $HgCl_2$ for 5 min(S_2),Ethanol for 10 sec (S_3),10% sodium hypochlorite (NaClO) for 10 min + Ethanol for 10 sec (S_4) &0.1% $HgCl_2$ for 5 min + Ethanol for 10 sec (S_5) are the five sterilants used.

Table 2
Influence of sterilants and explants origin on explant survival (%) of cherry rootstock "Colt" (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind)

Explant origin (F)	**Sterilants (S)** S_1	S_2	S_3	S_4	S_5
F_1	20.33	15.93	12.90	**60.33**	13.26
F_2	50.66	13.13	30.33	22.93	10.30
C.D($P \leq 0.05$)			**Explant (F)**	0.43	
			Sterilants (S)	0.69	
			(F) X (S)	0.98	

The data was analysed by comparing means using one-way ANOVA, and the significance was determined by CD $_{(p \leq 0.05)}$ using OPSTAT(v.6.8).Forced shoot tips(F_1) and Unforced shoot tips(F_1) are the two explants types and 10% sodium hypochlorite (NaClO) for 10 minutes (S_1), 0.1% $HgCl_2$ for 5 min(S_2),Ethanol for 10 sec (S_3),10% sodium hypochlorite (NaClO) for 10 minutes + Ethanol for 10 sec (S_4) &0.1% $HgCl_2$ for 5 min + Ethanol for 10 sec (S_5) are the five sterilants used.

Table 3
Influence of growth regulators, media and explants source on established cultures (%) of cherry rootstock "Colt" (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind)

Plant Growth Regulators (mg/l) (P)	BA (0.20)		BA (0.40)		BA (0.60)		BA + IBA (0.20 + 0.01)		BA + IBA (0.40 + 0.01)		BA + IBA (0.60 + 0.01)	
Media(M)\ Explant (F)	MS (M_1)	WPM (M_2)	MS (M_1)	WPM (M_2)	MS (M_1)	WPM (M_2)	MS (M_1)	WPM (M_2)	MS (M_1)	WPM (M_2)	MS (M_1)	WPM (M_2)
F_1	**75.00**	**81.00**	**65.00**	**81.00**	**83.33**	**75.00**	**61.00**	**65.33**	**57.00**	**63.00**	**68.33**	**64.33**
F_2	64.33	75.66	54.66	75.33	76.66	64.00	57.66	61.66	46.66	58.66	59.66	56.33

C.D($P \leq 0.05$)		
	Plant Growth Regulators (P)	0.50
	Media(M)	0.29
	Explant (F)	0.29
	(P) X(M)X(F)	1.00

The data was analysed by comparing means using one-way ANOVA, and the significance was determined by $CD_{(p \leq 0.05)}$ using OPSTAT(v.6.8).Forced shoot tips(F_1) and un-forced shoot tips(F_1) are the 2 explants types, MS and WPM are the two media and IBA & BA (0.20),(0.40),&(0.60),BA + IBA (0.20 + 0.01), (0.40 + 0.01),and (0.60 + 0.01) mg/l are two growth regulator in six combinations.

Table 4
Influence of growth regulators and media on proliferation (%) in forced explants of cherry rootstock "Colt" (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind)

Plant Growth Regulators (P) (mg/l)	M_1 (MS)	M_2 (WPM)
BA (0.20)	82.00	87.00
BA (0.40)	72.33	85.00
BA (0.60)	**92.00**	76.16
BA + IBA (0.20 + 0.01)	79.00	81.33
BA + IBA (0.40 + 0.01)	68.66	80.66
BA + IBA (0.60 + 0.01)	86.33	71.66
C.D($P \leq 0.05$)	Plant Growth Regulators (P)	0.78
	Media (M)	N/A
	(P) X (M)	1.10

The data was analysed by comparing means using one-way ANOVA, and the significance was determined by CD $_{(p \leq 0.05)}$ using OPSTAT(v.6.8). MS and WPM are the two media and IBA & BA (0.20),(0.40),&(0.60),BA + IBA (0.20 + 0.01), (0.40 + 0.01),and (0.60 + 0.01) mg/l are two growth regulator in six combinations

Table 5
Influence of growth regulators and media on number of shoots in forced explants of cherry rootstock "Colt" (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind)

Plant Growth Regulators (mg/l) (P)	**M_1 (MS)**	**M_2 (WPM)**
BA (0.20)	3.83	**5.83**
BA (0.40)	4.33	3.30
BA (0.60)	4.16	3.63
BA + IBA (0.20 + 0.01)	3.16	5.06
BA + IBA (0.40 + 0.01)	3.88	2.85
BA + IBA (0.60 + 0.01)	3.06	2.91
C.D(P ≤ 0.05)	**Plant Growth Regulators (P)**	0.25
	Media (M)	0.14
	(P) X (M)	0.35

The data was analysed by comparing means using one-way ANOVA, and the significance was determined by CD $_{(p \leq 0.05)}$ using OPSTAT(v.6.8). MS and WPM are the two media and IBA & BA (0.20),(0.40),&(0.60),BA + IBA (0.20 + 0.01), (0.40 + 0.01),and (0.60 + 0.01) mg/l are two growth regulator in six combinations

Table 6
Influence of growth regulators and media onshoot length (mm) in forced explants of cherry rootstock "Colt" (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind)

Plant Growth Regulators (P)(mg/l)	M_1 (MS)	M_2 (WPM)
BA (0.20)	13.16	**16.16**
BA (0.40)	10.26	9.06
BA (0.60)	10.16	7.70
BA + IBA (0.20 + 0.01)	12.33	14.16
BA + IBA (0.40 + 0.01)	9.16	8.43
BA + IBA (0.60 + 0.01)	8.26	6.76
C.D($P \leq 0.05$)	Plant Growth Regulators (P)	0.28
	Media (M)	0.16
	(P) X (M)	0.40

The data was analysed by comparing means using one-way ANOVA, and the significance was determined by CD $_{(p \leq 0.05)}$ using OPSTAT(v.6.8). MS and WPM are the two media and IBA & BA (0.20),(0.40),&(0.60),BA + IBA (0.20 + 0.01), (0.40 + 0.01),and (0.60 + 0.01) mg/l are two growth regulator in six combinations

Table 7
Influence of growth regulators and media onRooting(%) in forced explants of cherry rootstock "Colt" (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind)

Plant Growth Regulators (P)(mg/l)	M_1 (MS)	M_2 (WPM)
IBA (0.50)	32.66	55.66
IBA (1.00)	**90.66**	23.66
IBA (1.50)	24.33	45.66
IBA (2.00)	85.66	20.00
C.D($P \leq 0.05$)	Plant Growth Regulators (P)	1.91
	Media (M)	1.35
	(P) X (M)	2.71

The data was analysed by comparing means using one-way ANOVA, and the significance was determined by CD$_{(p \leq 0.05)}$ using OPSTAT(v.6.8).MS and WPM are the two media and IBA (0.50),(1.00),&(1.50)(2.00) is the growth regulator in four combinations

Table 8
Effect of growth regulators and media onnumber of roots in forced explants of cherry rootstock "Colt (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind)

Plant Growth Regulators (P)(mg/l)	M_1 (MS)	M_2 (WPM)
IBA (0.50)	1.87	3.81
IBA (1.00)	**5.92**	0.99
IBA (1.50)	1.71	2.73
IBA (2.00)	4.75	0.91
C.D($P \leq 0.05$)	Plant Growth Regulators (P)	0.03
	Media (M)	0.02
	(P) X (M)	0.04

The data was analysed by comparing means using one-way ANOVA, and the significance was determined by $CD_{(p \leq 0.05)}$ using OPSTAT(v.6.8).MS and WPM are the two media and IBA (0.50),(1.00),&(1.50)(2.00) is the growth regulator in four combinations

Table 9
Effect of growth regulators and media on root length (mm) in forced explants of cherry rootstock "Colt" (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind)

Plant Growth Regulators (mg/l) (P)	M_1 (MS)	M_2 (WPM)
IBA (0.50)	27.66	42.63
IBA (1.00)	**70.16**	21.00
IBA (1.50)	21.46	40.10
IBA (2.00)	61.63	18.66
C.D($P \leq 0.05$)	Plant Growth Regulators (P)	0.86
	Media (M)	0.61
	(P) X (M)	1.22

The data was analysed by comparing means using one-way ANOVA, and the significance was determined by $CD_{(p \leq 0.05)}$ using OPSTAT(v.6.8).MS and WPM are the two media and IBA (0.50),(1.00),&(1.50)(2.00) is the growth regulator in four combinations

Table 10
Effect of growth regulators and media on *ex vitro* survival (%) in forced explants of cherry rootstock "Colt"

Desired combination	H_1 (Cocopeat)	H_2 (Sand)
MS + IBA (0.50mg/l)	74.97	58.33
MS + IBA (1.00mg/l)	41.66	25.00
MS + IBA (1.50mg/l)	50.00	33.33
MS+ IBA (2.00mg/l)	25.02	16.69
C.D($P \leq 0.05$)	Plant Growth Regulators (P)	19.89
	Media (H)	14.06
	(P) X (H)	N/A

The data was analysed by comparing means using one-way ANOVA, and the significance was determined by $CD_{(p \leq 0.05)}$ using OPSTAT(v.6.8).MS is the growth medium, IBA (0.50),(1.00),&(1.50)(2.00) is the growth regulator in four combinations and H_1 (Cocopeat) & H_1 (Sand) are the two hardening media

Discussion

Culture asepsis (%), Explant survival (%) and Culture establishment (%)

The present research has been done to standardize a protocol on *the in vitro* propagation of cherry rootstock 'Colt' (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind). As depicted in Table 1, during sterilization regime maximum culture asepsis (%) was obtained in unforced shoot tips(F_2) with 0.1% $HgCl_2$ for 5 minutes followed by Ethanol for 10 seconds (S_5) (60.66%) which is supported by various researchers. In 'Oblacinska' rootstock of sour cherry, a minimum rate of contamination (up to 27%)was noticed in unforced (dormant buds) collected during November and December[17].In peach explants,maximum aseptic cultures 63.34% were obtained with 70% ethyl alcohol for 30 seconds along with 0.1% $HgCl_2$ 3 minutes and 1% NaOCl for 3 minutes [21].In quince (*Cydonia oblonga*)cv. SKAU-016optimum culture asepsis (55.99%) has been noticed with 0.1% $HgCl_2$ for 10 minutes along with 70%ethanol for 10 seconds[22]. In sweet cherry (*Prunus avium* L.) rootstocks Gisela 12 and Maxma 14, more culture asepsis (%), was obtained in commercial bleach (25%) and 0.01% and 0.03% of HgCl2 (*w/v*) [20]. Low rates of contamination (26.58%) has been found in 0.1% HgCl2 and 70% Ethyl alcohol (70%) for 4 minute in (*Musa paradisiaca* L.) var "Udhayam"[23]. The mixture of 0.1% HgCl2 for 10 min and 70% ethyl alcohol showed maximum success during sterilization in all three explants *viz*., shoot tip (89.58%), nodal segment (89.57%) and leaf explants (83.33%) [24]. A study was conducted by Sawant et al [25], where a protocol on surface sterilization was developed for 22 varieties of rice (*Oryza sativa* L.) using 1.0% $HgCl_2$ for 10 min followed by 70% ethyl alcohol for 1 minute with 86.67% aseptic cultures. As in this experiment Table 2, maximum explant survival (%) (60.33%) was obtained in forced shoot tips (F_1) with 10% sodium hypochlorite (NaOCl) for 10 minutes followed by Ethanol for 10 seconds (S_4) which is supported by Dar et al., 2010 [26] as in sour cherry (*Prunus cerasus* L.) rootstock, forced dormant cuttings showed higher rates of survival. In the forced explants in sweet cherry (*Prunus avium* L.) rootstock "Mazzard" showed maximum survival rate (Peer et al., 2011 [27]. The survival (90%) in apricot (*Prunus armeniaca*L.) is affected via 1.5% NaOCl for 10 minutes [28]. The forced explants in sweet cherry (*Prunus avium* L.) cv. "Bigarreau Noir Grossa" showed maximum survival rate [29]. Also,Mihaljevic*et al*. [30] showed that NaOCl gave explants survival (80%) in "Oblacinska" sour cherry.

Wolella [31] showed survival rate (97%) with 2% NaOCl for 15 minutes during in *vitro* studies on plum (*Prunus domestica* L.) *cv. Stanley.* In cherry rootstock CAB-6P, maximum explants survival (97%) was noticed with NaOCl treatment [14]. A protocol was developed to sterilize nodal ex-plants in thirteen peach accessions viz., Flame crust, Early green, Florida king, Spring crust, 669 No.2., No.2, Early grand, A7, Clark king, 4.5, Chaki pakora, A4 and A2. During this study it was found that NaOCl 28% for 15 minutes and 70% Ethyl alcohol for 1minute gave complete survival (100%) in four accessions viz., 669 No.2, Early green, 4.5, A7,and Clark king as reported by [32]. In *Juglans regia* L. cv. Sulaiman(Persian walnut) forced shoot tips depicted highest rates of survival (74.5 ± 2.4%) [33]. As in this experiment Table 3, highest establishment percentage (83.33%) with MS medium, BA (0.6) mg/l & in forced shoot tips (F_1) depicted in Fig. 1a which is supported by findings of [34] who found that during *in vitro* culture in four sweet cherry (*Prunus avium* L.) cultivars maximum bud establishment of buds was inMS medium along-with BAP 1 mg/liter. Pruski[35] showed that during *in vitro* establishment of cultures in Mongolian cherry (*Prunus fruticosa* L.), and Nanking cherry (*Prunus tomentosa* L.), MSMO (Murashige and Skoog Minimal Organic) solid medium fortified with either 4.44 or 8.88 µM 6-benzyl amino purine (BA), gave superior results. In MS medium supplemented with 1.5 mg/L BAP (6-Benzylaminopurine) highest culture establishment has been found on in rough lemon (*Citrus jambhiri*Lush)[36]. Farag *et al*.[28] reported that MS medium was best for culture establishment in apricot (*Prunus armeniaca*L.). In pomegranate, highest culture establishment (68.5%) has been found in MS medium treated with 1.0 mg/L BAP (Singh and Patel 2016) [37]. Thakur et al., 2016 [38] reported that during *in vitro* propagation in Gisela-5 rootstock (*Prunus cerasus* x *Prunus canescens*) 0.1% HgCl2 for 5 minutes has been found best for establishment of buds July (70%) which is followed by February (51.50%), compared to 50(%) in May on MS medium fortified with 0.5 mg/l BAP. Lizzarraga et *al.* [39] found that in 8 cultivars of apple culture establishment (%) protocol was developed using MS medium fortified in BA (1.0 mg/l). The highest establishment of cultures was obtainedwith MS medium augmented with 1.5 mg/l of BAP using tips of shoots (97.90%) and segments of nodes (95.74%) respectively [24].

Shooting proliferation

Bigarreau Noir Grossa

Significantly higher shoot proliferation (92.00%) is obtained with MS medium with 0.6 mg/l BAP (Table 4) supported byLarraburu*et al*[40] who used BA with MS medium for *in vitro* studies of Photinia maximum proliferation success was found for 24 months. In Jojoba maximum internodal and apical cuttings proliferate on MS medium [41]. In chokeberry during *in vitro* culture, MS medium has been used for initiation and proliferation respectively[42]. In *in vitro* studies of paradise tree (*Melia azedarach*) maximum multiplication rate (of micro-shoots) was observed on MS medium amended with BAP(0.5 mg/l) [43]. Ray *et al*[44] reported that MS basal medium fortified with different plant growth regulators combinations upon various stages of development in *Cordyline terminalis*gave maximumregeneration (95 ± 2.8). Renu and Singh [45] noticed highest proliferation of shoots using MS basal medium in Red Fuji cultivar of apple (*Malus* × *domestica*Borkh.) InMS medium, highest multiplication of shoots was found in Gisela 12 followed by Maxma14 (6.2 shoots/explant &4.4 shoots/explant respectively) [20].

Hosseinpour*et al*[15] reported that in M × M60 cherry rootstock BAP is important for on proliferation and elongation in shoots. During tissue culture of Gisela 5' cherry rootstock,using BA(0.5mg/l) highest proliferation rates were found [46]. Since N6-benzyladenine (BA) is a cytokinin used mostly during initiation and proliferation stages of 'Gisela 5' rootstock of cherry at varying concentrations [47, 48, 49, 38].

Maximum shoot number (5.83) in WPM medium with BA (0.2) mg/l (Table 5) & maximum shoot length (16.16 mm) in WPM medium with BA (0.2) mg/l (Table 6) Fig. 1b as supported by Sisko[50] that reported WPM resulted in the maximum rate of shoot multiplication in 'Gisela 5' cherry rootstock (4.2 shoots/explant).Agahye*et al*[51] found that in Gisela 6, semi-dwarf rootstock of cherry WPM media showed maximum height of shoots. Fallapouret al[52]reported similar results whilemicropraopagation of 'Gisela 5' (rootstock of cherry); WPM showed maximum number of shoots. N6-benzyladenine (BA) is one of the effective cytokinin for elongation of shoots in various woody plants *viz.*,Saponari*et al.* [53] in *Prunus cerasus*, Mansseri-Lamrioui*et al*[54] in*Prunus insigna*, and Sharma *et al*[55] in *Prunus avium*.

Rhizogenesis and Hardening

Significantly higher rooting percentage (90.66%) was recordedusing MS basal medium with 1.00 mg/l of IBA (Table 7). Highest root number (5.92) is obtained in MS medium supplemented with 1.00 mg/l of IBA (Table 8). Also, maximum root length (70.16 mm) was reached in MS medium augmented with1.00 mg/lof IBA (Table 9) Fig. 1c. This is in conformity with study of various workers for *in vitro* propagation of different rootstocks of cherry [47, 50, 56, 57, 58, 59]. Muna *et al* [60] found highest rate of rooting (%) Maxama-14 rootstock of cherry at minimal IBA levels MS medium. In *Diospyros kaki* L. *cv*. "Kaki tipo" there is culture of shoots in growth medium containing IBA (1 mg/l) [61]. Similarly, Mir *et al*[62] and Aghaye*etal*[51] depicted that during *in vitro*-rhizogenesis in ½ MS medium fortified in IBA in Mazzard, Mahaleb and Gisela 6 cherry rootstocks. In jojoba maximum rooting is found in ½ MS medium with 14.7 µ M IBA [41]. In paradise tree (*Melia azedarach*) maximum rooting was found in MS medium with IBA (0.1 mg/l) during*in vitro* studies [43].Also, in Tetra (*Prunus empyrean*) rootstock of cherry maximum rooting was found in medium with IBA than IAA [63]. In ½ MS medium with IBA (1 mg/l), sour cherry (*Prunus cerasus* L.) 'Oblacinska' rooting percentages 71.3% and 81.3% was obtained in 'OV 17' and 'OV 32'accessions, respectively, [17].During tissue culture incherry birch (*Betulalenta* L.), it was found that 80% of shoots formed roots in ½ MS medium fortified with 10 µM of IBA [64]. *In vitro* rhizogenesis in clonal rootstock of cherry Gisela 5 (*Prunus cerasus* × *Prunus canescens*), showed maximum rooting (100%) on IBA (0.5 mg/l) with MS medium[6]. During *in vitro* rooting of MA×MA60 semi-dwarf sweet cherry rootstock, the maximum rooting percentage (75%) and number of roots (5.33) was found in MS medium [18]. Under *in vitro* studies in Gisela 6 semi-dwarf cherry rootstock, maximum rooting (100%) was in Gisela 12 and (88.88%) Maxma 14 in half MS + 1.00 mg/l of IBA [20].During this experiment, it was found that maximum *ex-vitro* survival (%) was observed in plantlets hardened with (74.97%) success in cocopeat (Table 10) Fig. 1d. Our research is favoured by various authors viz., Kumar *et al*[65] in apple cv. Golden Delicious, Cuko*et al*[66] in two cvs. of apple *viz*., Golden Delicious and Starking Delicious, Batool *et al* [67] in banana, Kumar *et al*[6]in Gisela 5 (cherry rootstock) & Mon *et al*[68] in banana with superior survival (%). Coco peat refers to fiber of coir with pith of husk of coconut at base giving ventilation to roots for proper growth [69]. Cocopeat has pores which aid in to give channels for aeration and drainage [70].Modgil and Sharma[71] observed maximum 95 (%) success with peat during hardening of apple rootstock Malling 7.100% coco peat resulted in better survival rates (80%) during *in vitro*growth in *Eucalyptus camaldulensis*[72]. Well rooted plantlets were hardened with cocopeat in 'Bhagwa' variety of pomegranate with excellent results[70]. The survival rate (100%) was found in coco peat (100%) during hardening of gerbera (*Gerbera jamesonii* Bolus) [73].

Conclusions

The current research demonstrates the optimized protocol for *in vitro* propagation of 'Colt' (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind) rootstock of cherry.This protocol was established in the shortest period of 3 months

time. In summary, maximum culture asepsis (%) obtained in unforced shoot tips(F_2) with 0.1% $HgCl_2$ for 5 minutes + Ethyl alcohol for 10 seconds (S_5) (60.66%), maximum explant survival (%) (60.33%) was obtained in forced shoot tips (F_1) with 10% sodium hypochlorite (NaOCl) for 10 minutes + Ethyl alcohol for 10 seconds (S_4) and highest establishment percentage (83.33%) with MS medium, BA (0.6) mg/l & in forced shoot tips (F_1). Significantly higher shoot proliferation (92.00%) is obtained with MS medium with BA (0.6) mg/l, maximum shoot number (5.83) in WPM medium with BA (0.2) mg/l & maximum shoot length (16.16 mm) in WPM medium with BA (0.2) mg/l.Significantly higher rooting percentage (90.66%) were obtained in MS medium with IBA (1.00) mg/l ,highest root number (5.92) is obtained in MS medium with IBA (1.00) mg/l.Also, maximum root length (70.16 mm) is obtained in MS medium with IBA (1.00) mg/l. In this experiment, it was found that maximum *ex-vitro* survival (%) was observed in plantlets hardened with (74.97%).

Materials & Methods

Sterilization &Preparation of plant material

The current experiment was performedin the Tissue Culture Laboratory, Division of Fruit Science, SKUAST-Kashmir, Shalimar. The current study used 2classes of explants: forced and unforced. The forced wood was harvested from mature standing stock plants of "colt" from the start of dormancy in November to December. To force the dormant wood 15–20 cm long shoot segments with latent buds (subterminal or apical potion)with 10–15 mm diameter were excised from "colt" cherry trees were in

bearing stage. This dormant wood was pre-treated with Captan at 0.2% concentration before being stored in poly-bags at 4 ± 3 ◦C until further use. Forcing was carried out in accordance with the procedure outlined by Dalal*et al*[74]. During the active growing season, active shoot tips (unforced) of 2–3 cm in length were taken from mature cherry rootstocks of "colt" grown in the experimental block of SKUAST-K, Shalimar. Unforced explants were placed in flasks containing normal tap water and transported to lab, where they were washed vigorously and kept under running water for at least 1 hour. In this experiment, five types of sterilants were used to sterilize explants: 10% sodium hypochlorite (NaOCl) for 10 minutes (S1), 0.1% HgCl2 for 5 min (S2), ethanol for 10 sec (S3), (S1)+ (S3) & (S2)+ (S3). Percent culture asepsis & Explant survival (%) was estimated four weeks after sterilization as follows:

Explant survival (%) = (number of explants survived/total number of explants sterilized) x 100

Culture asepsis (%) = (number of aseptic explants obtained /total number of explants sterilized) x 100

Culture media and culture conditions

After sterilization with $HgCl_2$/NaOCl the explants were flushedin sterile water, patted dry with sterile tissue paper and cut with a sterile blade into 1.5-2.0 cm cuttings with a single bud. The prepared segments were then inoculated on MS medium [75] or Woody plant medium [76] containing macro and micro-salts, and vitamins for establishment. Sucrose was kept at a concentration of 3%, myo-inositol at 100 mg/l, and Plant Growth Regulators (PGR's) [auxins and cytokinins]at different concentrations and combinations viz; BA (0.20), (0.40)&(0.60) & BA + IBA concentrations of (0.20 + 0.01), (0.40 + 0.01) and (0.60 + 0.01) mg L^{-1}. Agar was used to solidify the media, which was kept at 5.7 pH.The culture vessels (test tubes or flasks) containing the prepared media were then sterilized in an autoclave at 121°C temperature and 15 psi pressure for 15–20 minutes. The cultures were incubated under controlled temperature and light conditions in a culture/growth room at 24°C &light:dark cycle of 16:8 hours. After 30 days of inoculation, the percentage of established cultures was calculated using the best type of explant for future experiments.

Established cultures (%) = (number of explants cultures established/total number of cultures inoculated) x 100

The sterile forced shoot tip cultures that showed successful establishment on the primary culture medium were further split into nodal segments (0.5-1.0 cm long) for subculture.Such segments were carefully inserted on MS & WPM culture medium with BA (6-Benzylamino Purine) for proliferation atBA (0.20),(0.40),& (0.60); BA + IBA (0

.020 + 0.01), (0.40 + 0.01), and (0.60 + 0.01) mg/l. The proliferation coefficients, plus the number and length of shoots (mm), were calculated after post 4-week interval during which the cultures were maintained at a temperature of 25°C, an illumination level of 2000 Lx and light/dark cycles of 16/8 h.

Proliferation percentage (%) = (number of buds regenerated /number of buds inoculated) × 100

The sterile proliferated shoot tip cultures were rhizogenized in different media (MS & WPM) with different levels of IBA (0.50), (1.00), (1.50), and (2.00) mg/l, respectively. The rooting coefficients, as well as the number and length of roots (mm), were calculated post 4-week interval during which the cultures were maintained at a temperature of 25°C, an illumination level of 2000 Lx and light/dark cycles of 16/8 h.

Rooting Percentage (%) = (number of explants rooted/number of explants inoculated) × 100.

Acclimatization

The successfully developed plantlets were carefully removed from the culture flasks using long forceps preventing any injury to plant and washed with sterile water to remove the agar adhering to roots. These plantlets were treated with 0.2 gl-1 Mancozeb solution (Aria, Iran) and placed in covered jars with 100% cocopeat and sand. The pot tops were wrapped with clear plastic, and

they were grown in a shaded poly-house at 23°C, and to maintain humidity (90%) they were sprayed with water once a day. The plastic covers were removed after three weeks of plantation and the delicate plantlets were watered twice daily. The plants were transferred to larger pots after adaptation, with the same mediumwhich were then moved into a shade house with indirect sunlight &lower RH.

Survival percentage = (number of the plants surviving after transplantation/number of plantstransplanted) × 100.

Data Analysis

The data recorded for different parameters during the entire course of experimentation were statistically analyzed using OPSTAT (v.6.8) under a completely randomized design (CRD) with three replications.

Declarations

- **Ethics approval and consent to participate**: We declare that experimental research on cherry plants were done in compliance of institutional, national, and international guidelines and legislation. Seeds of cherry were obtained from the Germplasm Resources var., colt were done in accordance with the guidelines set by ICAR-India. All authors have agreed upon having participation in the said research
- **Consent for publication**: Not Applicable
- **Availability of data and materials**: The full dataset generated and analyzed during the current study is available in the SKUAST-Kashmir India
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Figures

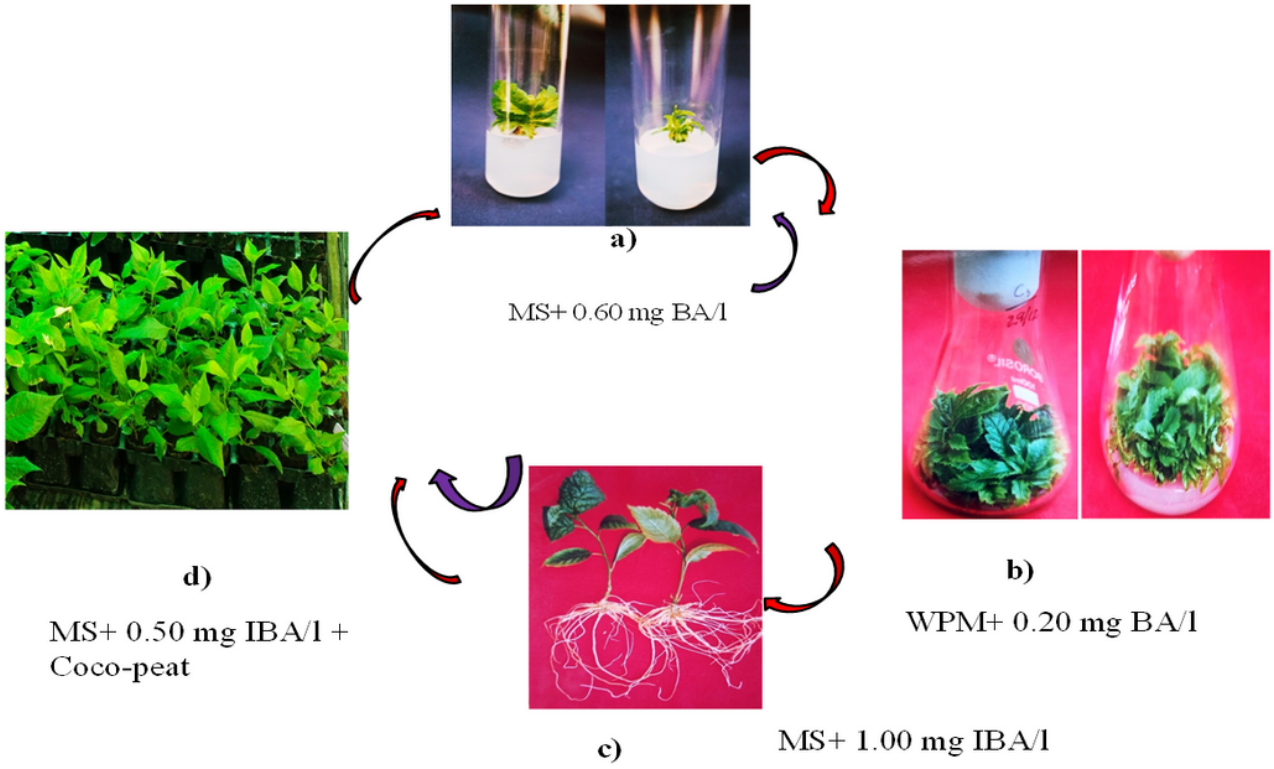

Figure 1

(a,b,c,d): *In vitro* propagation of 'Colt' (*Prunus avium* F 299/2 x *Prunus pseudocerasus* Lind) rootstock of cherry ; a) Culture Establishment , b) Shooting , c) Rooting, d) Hardening