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Resolving the browning during the establishment of the *in vitro* propogation of *Prunus avium* cv. 'Fuchen'

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Abstract: *Prunus avium* cv. 'Fuchen' is a cultivated species with a high-profile economic benefit. Four media were used as a basal media equipped with four exogenous hormones to establish an intact regeneration system. Sodium thiosulfate ($Na_2S_2O_3$) and active carbon (AC) improved the browning phenomenon. The results indicated that 1.0 g/L of AC and 30 g/L of $Na_2S_2O_3$ were the best combination to inhibit the browning on the optimal basal medium – olive medium (OM) (followed by woody plant medium). The OM containing 1.5 mg/L of zeatin, 2.0 mg/L of α-naphtalenaecetic acid (NAA) and 0.5 mg/L of kinetin was beneficial for the germination and growth of the axillary buds. Green bud points were visible at the base of the swollen base after 25 days with a high proliferation coefficient (more than 7.50). The most optimal medium for rooting was ½ OM combined with 1.0 mg/L of NAA and 0.5 mg/L of indole-3-butyric-acid with a 100% rooting rate. The survival rate was up to 100% after 60 days with acclimatisation. Generally, a high-effective regeneration system was established, which provides a reference for keeping the excellent traits of the cultivar.

Keywords: sweet cherry 'Fuchen'; bud proliferation; basal medium; rapid propagation; browning inhibition

The sweet cherry (*Prunus avium* L.) is a popular and important commercial fruit tree in many temperate countries of the world (Sgamma et al. 2015; Welk et al. 2016). Till now, botanical species have been propagated through different propagation methods including seeding, grafting and cutting. Amongst these methods, grafting uses clonal propagation which uses the rootstock for modification of the growth, quality and other desired properties of commercial varieties, thereby obtaining more excellent properties. These cultivars have different commercial values due to the difference in the chemical components and nutrients. The dietary phenolic components fluctuate with cultivated varieties. Furthermore, the antioxidant properties would produce some differences among these species (Martini et al. 2017). Moreover, there also exists an obvious distinction in the flavour owing to the variation in the sugar, pH value, hardness, and colour parameters (Usenik et al. 2008; Hayaloglu, Demir 2015, 2016). Mature fruits can be widely processed into various forms, like jams, wine, and can be dried, candied,

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and are characterised by the attractiveness of the colour, clean appearance, firmness and deliciousness (Budak 2017). These processed products have other beneficial properties to human beings, such as enhancing the sleep quality (Howatson et al. 2012) and having potential anti-inflammatory abilities (Šarić et al. 2009).

P. avium cv. 'Fuchen' is a hybrid variety from the progeny of the parental combination of Summit × Hongdeng developed at the Fruit Trees Research Institute, Yantai Academy of Agricultural Sciences, Shandong Province, China (Zhang et al. 2019). The tree is moderately vigorous having strong cold resistance. The fruits are early cropping, are roundish-cordate in shape, with a bright red ground skin colour and a slightly red sarcocarp. The dorsal suture of the mature fruit is glazy, which is similar to the characteristic of the Summit variety, with an average 9.7 g per fruit in weight. Due to their long-term storage period advantage, fruit trees have been introduced into Lijiang City of Yunnan Province. Usually, *Cerasus tomentosa* (Thunb.) Wall. is used to graft the sweet cherry cv. 'Fuchen' in order to maintain its heterosis as much as possible. However, some disadvantages exist with this method, such as a low survival rate, being susceptible to serious diseases (brown spot disease and gummosis), slow growth, and the influence of the rootstocks on any valuable advantages. Tissue cultures could maintain the excellent characteristics using suitable explants of cultivated species that are characterised by excellent quality having special genotypes. These certified genotypes could be excellently managed through *in vitro* tissue cultures with high multiplication rates. Researchers have obtained stable clonal plant materials for high economic profiles (Quambusch et al. 2016). Ružić and Vujović (2008) established a perfect micropropagation protocol for the sweet cherry cv. 'Lapins', a leading sweet cherry cultivar in the world assortment, using lateral buds with the help of a Murashige and Skoog (MS) medium and four cytokinins. Matt and Jehle (2005) developed a successful *in vitro* plant regeneration of five cultivars using leaves and internode sections, whose results indicated that the internode sections were more effective in obtaining higher shoot regeneration than those of the leaves. Some early studies mainly focused on *in vitro* organ regeneration using leaves as the explants, which failed by producing a small number of shoots per explant even though they tried their best to change the combination and dosage of the plant growth regulators (Grant et al. 2000; Bhagwat, David-Lane 2004).

The main objective of our study is to establish an integrated regeneration system of the sweet cherry cv. 'Fuchen' using internode sections combined with four media [MS, olive medium (OM), white medium (WM), and woody plant medium (WPM)] and four growth additives [zeatin, α-naphthaleneaceticacid (NAA), indole-3-butyric-acid (IBA), kinetin]. Meanwhile, we intend to eliminate the browning phenomenon which frequently occurs in the tissue cultures of the sweet cherry. The rooting seedlings can provide a reference for a high effective propogation rate of the cultivar.

MATERIAL AND METHODS

Preparation of plant materials. Strong individuals without pest and disease were selected as the research object, in which the juvenile nodal segments were collected in March, April and May of 2017. These nodal segments were soaked in a 10% (w/w) detergent solution (Common laundry detergent) for 10 min then washed for 30 min using tap water before being placed onto clean bench. The washed explants were surface disinfected with 75% (v/v) ethanol for 10 s followed by 6 min incubation in 0.1% (w/w) mercury bichloride as well as being rinsed five times in sterile water (at least 3 min each time). These single-node segments, after sterilisation, were excised to about 1.5 to 2 cm in length containing one third of the leaves for the *in vitro* propagation.

Initial culture. Four media were investigated to select the optimal medium for the tissue culture, including WPM, OM, WM and MS. Twenty grams per litre of sucrose and 4.6 g/L agar were added to all the media, except for 30 g/L of sucrose in the MS medium. The pH value of these media was adjusted to 5.4–5.6. All of the reagents and plant growth regulators were of analytical grade and purchased from Beijing Dingguo Biotechnology Co. Ltd. (Beijing, China). These prepared media were autoclaved at 121 °C for 22 minutes. The temperature of the culture room was controlled at 22 ± 1 °C and was provided with cool-white fluorescent tubes (40–60 μmol/m^2/s light intensity) under a 10 h photoperiod. Zeatin was used as a growth additive and the four media equipped with different dosages of sodium hyposulfite ($Na_2S_2O_3$) were regarded as factors, the combination experiment was investi-

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gated to select the optimal medium. Active carbon (1.0 g/L) was added to all the combination groups. Each group contained 20 bottles with eight explants in each bottle with three replicates per group. The explants were collected and disinfected within 2 hours. The sterile explants were inserted into the medium, and the same medium was changed every seven days. After three cycles, the survival rates and growth conditions were analysed to obtain the best medium and the most effective combination of controlling the browning.

Regeneration of nodal segments and proliferation culture. Based on the results of the initial culture, OM was used as the basal medium. An L_9 (3^3) orthogonal test was conducted via three factors (zeatin, NAA, and kinetin) combined with three levels (Table 1)**.** Each group contained 20 bottles with five explants in each bottle with three replicates per group. The proliferation coefficient was calculated after culturing for 50 days. The proliferation coefficient = the number of inoculations in the subculture/the total number of initial inoculations.

Induction culture of adventitious roots. ½ OM was used as the basal rooting medium equipped with different dosages of NAA and IBA according to a previous study (Xu et al. 2018). Strong seedlings (6–7 cm in length) were excised into 2–3 cm individuals from the 50-day proliferation culture and then cultured in the rooting media. Each group contained 20 bottles with five explants per bottle with three replicates per group. After 50 days, the rooting rate was evaluated to investigate the phytohormone influence under the different concentrations.

Acclimatisation and transplantation of rooting seedlings. The strong rooting seedlings were transferred into a greenhouse while keeping the culture bottles caps closed for a week. The seedlings, after removing the residual medium, were planted in a bubble chamber with peat and river sand (3:2, v/v) which was disinfected with 0.1% methanol (v/v). The temperature was kept at 20–25 °C and the humidity was maintained at 70% in the greenhouse for 60 days and then the plants were transplanted into a field.

Table 1. L_9 (3^3) orthogonal design for regeneration and proliferation of stem section

Levels	Factors (mg/L)		
	A (zeatin)	B (NAA)	C (kinetin)
1	1.0	0.5	0.1
2	1.5	1.0	0.5
3	2.0	2.0	1.0

NAA – α-naphtalenaecetic acid

Statistical method. An analysis of variance (ANOVA) was conducted using SPSS software (version 21.0). The significant differences were determined by the least significant differences test at a 5% probability ($P \leq 0.05$), the range analysis was calculated using the mean values.

RESULTS

The pre-experiment results indicated that browning occurred in 100% of the explants after three days and 10% of them survived after seven days. Further research showed that the survival rates were improved after adding active carbon or NaS_2O_3, in which the active carbon displayed a more effective impact to the initial culture. The browning phenomenon was completely removed after adding the combination of additives of active carbon and NaS_2O_3. In addition, significant differences existed in the survival rates of the different medium types ($P < 0.05$, Table 2). The OM groups showed a higher superiority than the other media (100% survival rate, Figure 1A),

Table 2. Effects of the basic medium/$Na_2S_2O_3$ combination on the browning and growth of the explants

No.	Basic medium	$Na_2S_2O_3$ (g/L)	Survival rates of explants (%)
CK	OM, WPM, MS, white	0	26.47 ± 1.011 57^{i}
B1	OM	30	100.00 ± 0.000 00^{a}
B2		40	95.00 ± 2.041 24^{b}
B3		50	81.88 ± 4.269 56^{c}
B4	WPM	30	93.13 ± 1.250 00^{b}
B5		40	80.33 ± 1.724 61^{c}
B6		50	76.25 ± 1.443 38^{d}
B7	MS	30	75.00 ± 2.041 24^{d}
B8		40	69.38 ± 2.393 57^{e}
B9		50	61.25 ± 1.443 38^{f}
B10	White	30	45.63 ± 1.250 00^{h}
B11		40	56.25 ± 4.330 13^{g}
B12		50	66.88 ± 2.393 57^{e}

MS – Murashige and Skoog medium; OM – olive medium; WPM – woody plant medium

$^{a–i}$Different lowercase letters indicate significant differences at $P \leq 0.05$ (LSD test)

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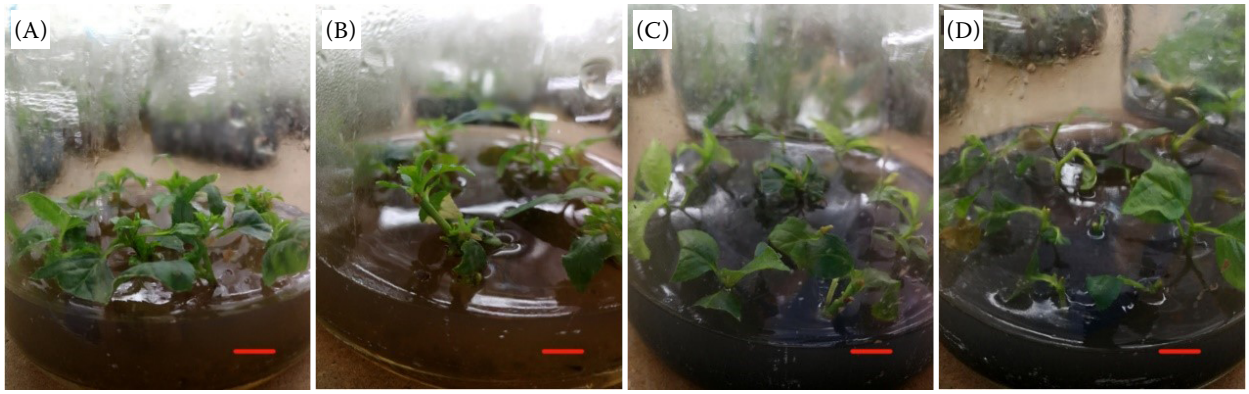

Figure 1. Browning inhibition and growth conditions cultured on different basal media
(A) B1 group, (B) B4 group, (C) B7 group, (D) B12 group, bars = 1.5 cm

especially when supplied with 30 g/L of NaS_2O_3, the growth conditions were excellent and there were healthy axillary buds. However, the survival rate increased as the NaS_2O_3 concentration increased and the growth of the sprouts became slow. This was followed by the WPM groups, in which the growth was less inferior than that of the OM groups, the survival rate was 93.13% when the growth conditions were excellent and the optimal dosage of NaS_2O_3 was 30 g/L (Figure 1B). There were high survival rates, slow growth, a few axillary buds in the MS and WM groups (Figures 1C and 2D). Therefore, OM was regarded as the optimal basal medium.

These plant materials, without browning, were cultured in the groups of the L_9 (3^3) orthogonal experiments, the dosage of the plant hormone and the results are displayed in Table 3. There were significant differences among the different dosage combinations in terms of the germination and growth of the axillary buds. The range analysis indicated that three plant hormones were effective in the regeneration and proliferation of the nodal segments. Zeatin plays a more vital role in the germination of the buds when compared with the range value of kinetin and NAA ($R_{ZT} > R_{KT} > R_{NAA} > R_{blank}$). The results of the one-way ANOVA are shown in Table 4, which can be interpreted by the fact that zeatin generated a significant positive impact to the proliferation coefficient between the concentration of 1.0–1.5 mg/L ($P < 0.05$), whereas NAA and kinetin did not show any significant influence ($P > 0.05$). Furthermore, Duncan's Test was conducted on three levels of the zeatin dosage (Level 1 = 1.0 mg/L, Level 2 = 1.5 mg/L, Level 3 = 2.0 mg/L). The comparison analysis indicated that the Level 2 dosage generated a more obvious effect to the proliferation coefficient, which displayed a significant difference compared with the Level 1 and Level 3 dosages (Table 5). Generally, the optimal combination of plant additives was $A_2B_3C_2$ (1.5 mg/L of ZT + 2.0 mg/L of NAA + 0.5 mg/L of KT; Table 1) for the regeneration and proliferation of the nodal segments in the sweet cherry cv. 'Fuchen'.

The axillary buds began to germinate after 10 days on the optimal media with the $A_2B_3C_2$ recipe (Figure 2A). The base of these axillary buds became intumescent with strong buds after 20 days (Figure 2B). The intumescent position began to germinate green bud points after 25 days (Figure 2C). There were many bud points on the intumescent base and these axillary buds grew into young stems containing nar-

Table 3. Regeneration and proliferation results of the stem section using the L_9 (3^3) orthogonal test

No.	Plant hormone (mg/L)				Propagation coefficient
	A (zeatin)	B (NAA)	C (kinetin)	blank	
P1	1.0	0.5	0.1	(1)	4.48
P2	1.0	1.0	0.5	(2)	4.84
P3	1.0	2.0	1.0	(3)	3.89
P4	1.5	0.5	0.5	(3)	7.48
P5	1.5	1.0	1.0	(1)	5.32
P6	1.5	2.0	0.1	(2)	6.92
P7	2.0	0.5	1.0	(2)	2.17
P8	2.0	1.0	0.1	(3)	3.01
P9	2.0	2.0	0.5	(1)	3.63
K_1	4.403	4.710	4.803	4.477	–
K_2	6.573	4.390	5.317	4.643	–
K_3	2.937	4.813	3.793	4.793	–
R	3.636	0.423	1.524	0.316	–

K – the mean; R – the range

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Table 4. Single factor analysis of the stem segment multiplication coefficient

Source	Type III sum of square	df	Mean square	*F*	*P*-value
A (zeatin)	20.085	2	10.043	14.889	< 0.05
B (NAA)	0.292	2	0.146	0.037	> 0.05
C (kinetin)	3.604	2	1.802	0.527	> 0.05
Blank	0.151	2	0.075	0.019	> 0.05

NAA – α-naphthaleneacetic-acid

Table 5. Duncan's test for three zeatin levels

Levels	Mean	0.05 level
2	6.573 3	a
3	4.403 3	b
1	2.936 7	b

[a,b]Different letters indicate significant differences at 5% level

row leaves after 30 days (Figure 2D and 2E). These bud points grew into young buds and the explants contained many buds and stems after 40 days (Figure 2F). The original cultured explants were covered by palingenetic buds surrounded by new-germinated bud points and the narrow leaves became oval in shape after 50 days (Figures 2G and 2H). The proliferation coefficient reached more than 7.50.

The induction results of the adventitious roots are displayed in Table 6. The rooting rate of the *in vitro* seedlings is zero in the blank group without any plant hormones. These plant materials could germinate roots in the NAA or IBA group with strong growth conditions, which could be interpreted that the germination of adventitious roots was easily affected by the exogenous hormones. There was not any significant difference among the different IBA concentrations combined with 0.5 mg/L of NAA. Whereas the rooting rate showed a significant difference in the different dosage NAA groups when the IBA concentration was the same ($P < 0.05$). These results can be interpreted as the NAA dosage played an important role in the induction of adventitious roots. A number of thick adventitious roots began to germinate from the base of the seedlings after 10 days (Figure 3A). There was a mass of axillary buds with root growth after 20 days and a developed root system formed with extended leaves after 30 days (Figures 3B and 3C). The survival rate

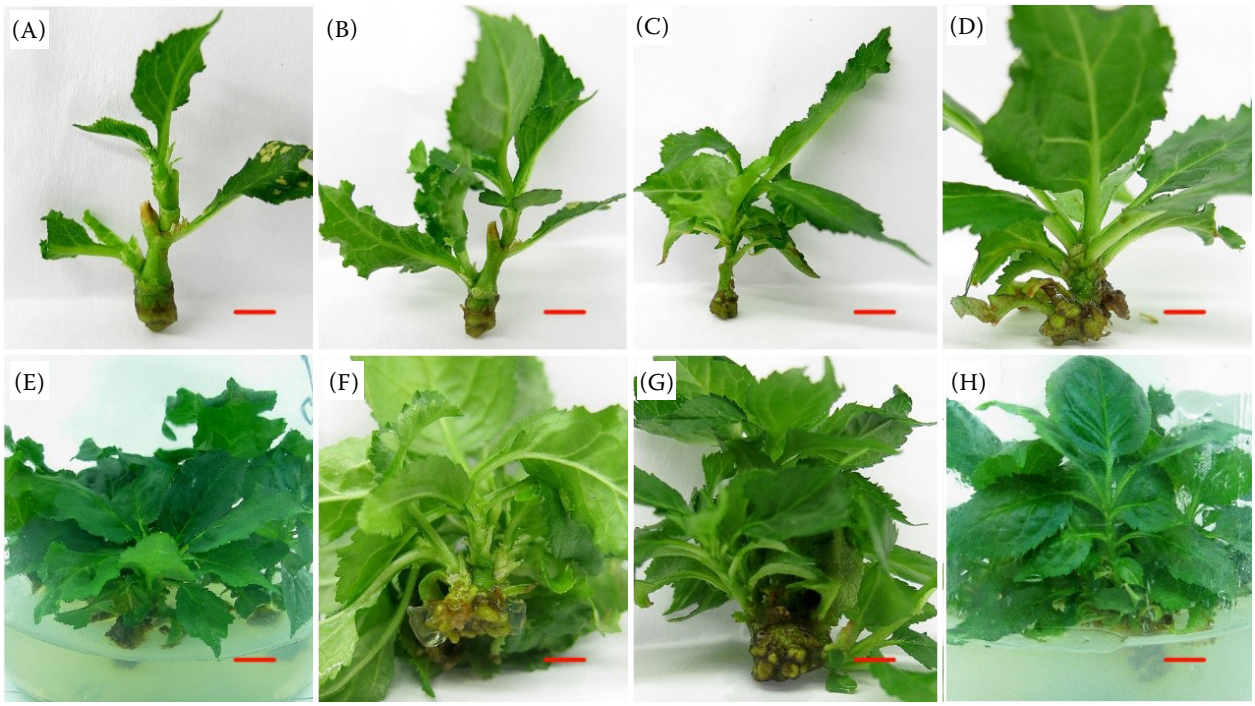

Figure 2. Regeneration and proliferation of the stem segments
(A) The axillary buds began to grow after 10 days; (B) the rapid-growth axillary buds and intumescent base after 20 days; (C) green young buds at the intumescent base after 25 days; (D and E) intumescent young buds and expanded leaves after 30 days; (F) plexiform bud from the green young buds at the intumescent base after 40 days; (G and H) cluster system with many branches and buds after 50 days. Bars A–D = 0.5 cm, bars E–H = 1.0 cm

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Table 6. Effects of the α-naphthaleneacetic-acid (NAA) and indole-3-butyric-acid (IBA) combination on the induction of adventitious roots

Medium	Hormone (mg/L)		Rooting rate
	NAA	IBA	
R 01	0	0	0.00 ± 0.000 00^{e}
R 02	0.5	0.1	44.75 ± 1.066 54^{cd}
R 03		0.3	43.00 ± 0.707 11^{d}
R 04		0.5	46.50 ± 0.500 00^{c}
R 05	1.0	0.1	98.00 ± 0.547 22^{a}
R 06		0.3	98.50 ± 0.223 61^{a}
R 07		0.5	100.00 ± 0.000 0^{a}
R 08	1.5	0.1	70.50 ± 1.024 70^{b}
R 09		0.3	70.50 ± 0.500 00^{b}
R 10		0.5	71.75 ± 1.066 54^{b}

$^{a–e}$Different lowercase letters indicate significant differences at $P \leq 0.05$ (LSD test)

was 100% after transplanting into the bubble chamber with peat and river sand (Figure 3D).

DISCUSSION

MS and ½ MS were the main media for the *in vitro* propagation of the sweet cherry in the published literature (Ružić, Vujović 2008; Quambusch et al. 2016). Additionally, there were other reports on tissue cultures of fruit trees using WPM (Bhagwat, David-Lane 2004), Quoirin/Lepoivre (QL), Driver and Kuniyuki (DK), Chee and Pool (CP), and their mixture media (Matt, Jehle 2005). Our results indicated that OM is the optimal medium for the propagation of the *P. avium* cv. 'Fuchen', in which the germinated explants extended the leaves and growth of the axillary buds. This was followed by WPM, where the growth was slower than the materials in the OM groups. In contrast to the published lit-

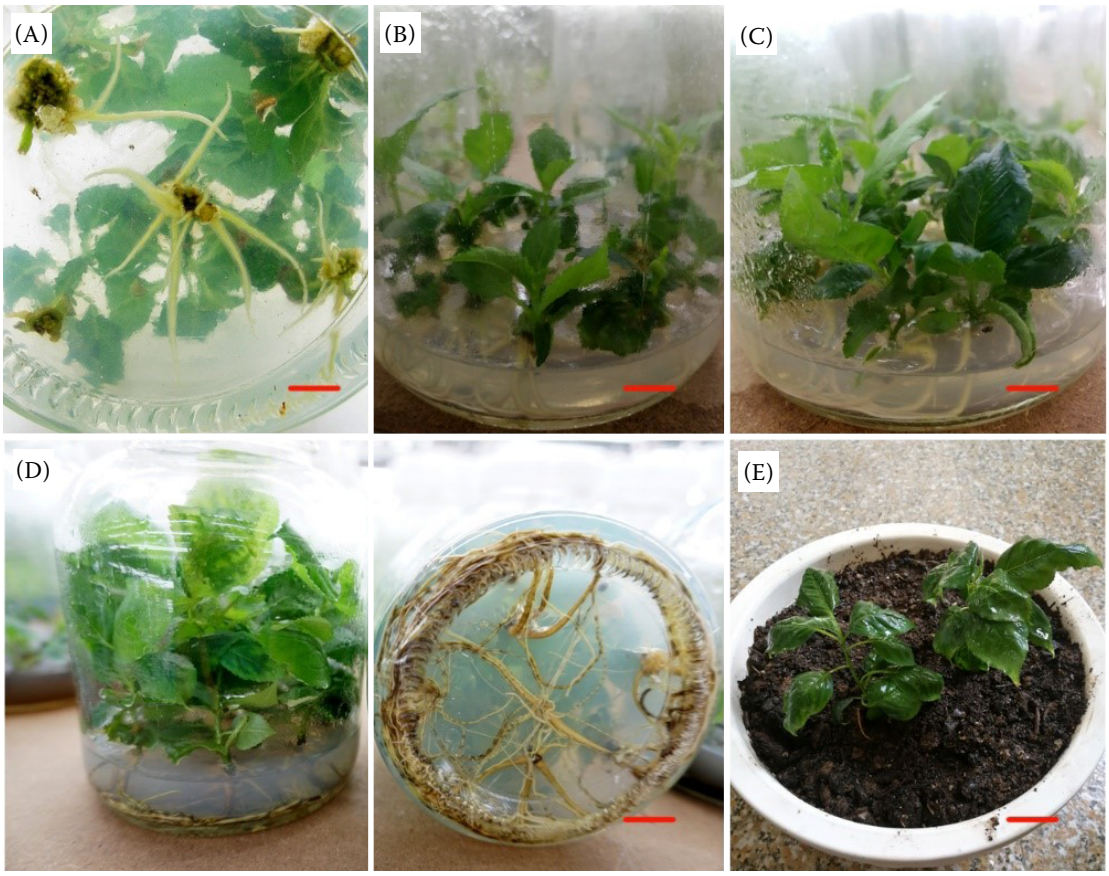

Figure 3. Induction of the adventitious roots and seedling domestication

(A) Adventitious roots began to germinate from the base of the stem segment after 10 days; (B) the growth condition of the tube seedlings after 20 days; (C) the growth condition of the tube seedlings and rooting after 30 days; (D) tube seedlings after 50 days; (E) seedling domestication. Bar A = 0.5 cm, bars B–D = 1.5 cm, bar E = 2.0 cm

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erature focusing on the tissue culture of cherries, MS was not the best basal medium where the cultured materials had slow growth with a long-period growth cycle. After further culturing, these young stems and leaves became yellow, and the leaf abscission phenomenon occurred at the basal stem while the bud tips were withered. On the one hand, this phenomenon can be interpreted as different varieties had different adaptability to the different basic media. On the other hand, OM contained a high content of mineral elements, which was beneficial for improving the growth conditions of the yellow leaves and weedy stems caused by the scarcity of elements, such as N, P, K, Zn, S. Moreover, OM contained biotin, folic acid, and glutamine which were absent in the other types of media. The high content of glutamine played an important role in transferring the ammoniacal nitrogen in the process of the sweet cherry cv. 'Fuchen' growth. We also conducted a culture group without glutamine, these plant materials grew slowly and tended to die after 60 days. The supplementary experiment group confirmed the vital impact of glutamine in the growth of the cultivar. According to previous studies, the basic culture medium has a great influence on the growth of different cherry varieties. Matt and Jehle (2005) found that DK/WPM (1 : 1) and QL were more stimulating to the occurrence of bud organs for the sweet cherry cv. 'Starking Hardy Giant', 'Schneiders', and 'Kordia', in a comparison to the DK, WPM, QL, CP, MS, DK and WPM media. Based on Bhagwat and David-Lane (2004), WPM was a more suitable medium for the plant regeneration of the sweet cherry cv. 'Lapins' and 'Sweetheart' than the MS medium. QL was successfully used for the bud regeneration and MS was used for the root culture in the sweet cherry cv. 'Vista', 'Tehranivee', 'Vouge' and 'Heidelfinger' (Canli, Tian 2008). Therefore, the selection of the basal medium is key for the further culturing the propagation of the sweet cherry 'Fuchen'. Otherwise, the function of plant hormone cannot come into play without the initial selection step.

Browning is a common phenomenon in the initial culturing of sweet cherries. For improving this undesirable problem, most researchers chose fresh plant materials (Li et al. 2014; Zhang et al. 2018a) or changed the basic medium (Ye et al. 2016). There was severe browning in the initial culture of the sweet cherry cv. 'Fuchen', which affected the survival rate of these explants. The survival rate was approximately 10% although the media were supplied with various plant hormones. Moreover, the successive transfer culture failed to eliminate the browning phenomenon. Our present study showed that active carbon and the antioxidant $Na_2S_2O_3$ could effectively inhibit the browning in the initial culture. There was not any browning in the groups supplemented with the antioxidant, which was the same as the results of the tissue culture of *Rubus subvacuums* (Guo et al. 2009). However, the high-dosage antioxidant decreased the survival rate, leading to speculation that there is a potential toxic action to the germination of young tissue. Herein, we suggested an effectively ancillary measurement to overcome the browning effect, named as the "Rapid Inoculating Method". The approach reduced the duration of the exposure between the culture materials and air, such as heating the wall of the culture bottle. Similar to our previous research of the strawberry cv. 'Akihime' (Zhang et al. 2018b), the browning disappeared in the transfer culture without the $Na_2S_2O_3$ or active carbon, when the phenomenon was removed in the initial culture and these plant materials were transferred in a timely manner with the growth cycle. It was interpreted that these plant materials can release the relative enzyme in a period of vigorous growth to control the browning.

There have been rare reports in the tissue culture of woody plants via an indirect organogenesis protocol (callus to adventitious buds), which may be caused by the biological properties of these trees that can accomplish dedifferentiation and fail to redifferentiate. The published literature is mainly concentrated on the germination of axillary buds using micro-cutting, a common method in the *in vitro* propagation of woody plants (Kappel et al. 2002; Ansar et al. 2009). It was found that the green bud points grew into adventitious buds on the swelling part of the basal stem (Figure 2). Different from the adventitious buds from the callus, the buds from the swelling part were rare and no-clustered, but they grew rapidly forming a strong and thick shape. The growth conditions have rarely been reported on in the research of sweet cherries till now. Li et al. (2015) thought that the buds at the basal stem of *Solanum pseudo-capsicum* belong to the indirect callus-mediated buds' organogenesis. The *in vitro* propagation of *Codonopsis bulleyana* also belonged to the indirect way (Wang et al. 2018). Ti et al. (2016) regarded the germination as a direct method, a growth of potential axillary buds in the tissue culture of *Halocnemum strobilaceum*. Different from the germination of *C. bulleyana*, these

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buds at the basal stem were germinated from septate internodes (without connection with the callus) after the base of stem became swollen when the axillary buds were luxuriant. Moreover, the germination position of these buds was uncertain, rather than from the potential axillary, which was different from the research of *H. strobilaceum*. In general, we speculate that these basal sweet cherry buds were germinated from the potential meristem (cambium) in the septate internodes of the base stems. The cells of the cambium conducted the cell division forming dedifferentiated cull clusters stimulated by the exogenous and endogenous plant hormones, which resulted in the swelling base. Finally, the potential adventitious buds broke though the cuticular layer forming strong buds, whose growth was similar to tiller seedlings, direct organogenesis in nature. In our present research of the sweet cherry cv. 'Fuchen', all of the axillary buds germinated and grew with the production of basal buds, which eventually formed a cluster system with vast buds and stems with the highest proliferation coefficient.

In this study, a relatively mature and complete tissue culture and rapid propagation system for the sweet cherry cv. 'Fuchen' was established, which can offer a new path for the preservation and rapid propagation of this excellent strain, and provide a reference basis and technical support for improvement of the variety. Meanwhile, this protocol can also lay an experimental foundation for the study of its genetic transformation.

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