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不同繁育方式对樱桃幼苗主要病毒病的影响

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摘 要:【目的】调查不同繁育方式获得樱桃苗木的病毒携带情况,为今后樱桃苗木最适繁育方式的选择以及苗木无毒化栽培管理提供理论指导。**【方法】**采取对角线五点取样法随机采集中国樱桃实生苗、樱桃砧木扦插苗、甜樱桃实生苗的样本各30份,樱桃砧木组培苗60份,采用RT-PCR法对6种病毒进行检测。**【结果】**6种病毒检测结果均呈阳性,经序列分析证明,6种病毒片段与GenBank中已登录的病毒核苷酸序列有较高一致性。本实验田间随机取样的中国樱桃实生苗、樱桃砧木扦插苗、樱桃砧木组培苗、甜樱桃实生苗的带毒率分别为86.67%、70.00%、0%和76.67%。中国樱桃实生苗样本植株带毒率为86.67%,单独侵染PNRSV的比例较高,为53.33%;樱桃砧木扦插苗的样本中樱桃坏死锈斑病毒(CNRMV)、李属坏死斑病毒(PNRSV)和樱桃绿环斑驳病毒(CGRMV)检出率分别为36.67%、26.67%和20.00%,这3种病毒樱桃扦插苗田间携带率较高;甜樱桃实生苗的田间带毒率为76.67%,其中95.66%为樱桃病毒A(CVA)单独侵染;从两个不同产区所取樱桃砧木组培苗(共60份样品)未检测到樱桃常见6种病毒。**【结论】**中国樱桃实生苗与砧木扦插苗的田间带毒率均较高,分别为86.67%和70.00%。甜樱桃种子不可默认为无病毒携带,反而极易感染与传播樱桃病毒A(CVA),并非无毒苗最佳繁育材料。

关键词:樱桃;苗木;病毒病;繁育方式

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Effects of different propagating patterns on the virus transmission of cherry seedlings

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Abstract:【Objective】At present, the cherry seedling market is disorderly, and the situation for virus carrying and spread through cherry seedlings propagated by different methods is unclear. This study investigated the virus carrying status of cherry seedlings by investigating different propagating methods, so as to provide theoretical guidance for the selection of the most suitable propagating methods for cherry seedlings, the virus-free cultivation and management of seedlings in the future.【Methods】Diagonal five-point sampling method was used to randomly collect Chinese cherry seedlings, including cherry rootstock cuttings, 30 samples of sweet cherry seedlings, 60 cherry rootstock tissue culture seedlings, and six types of viruses were tested by RT-PCR. One year old shoot with the same thickness was taken at the same location from each plant, and the phloem was placed into a liquid nitrogen tank for freezing, and stored in a −80 ℃ refrigerator. Total RNA was extracted from the samples, and six types of major cherry viruses were detected by RT-PCR. Total RNA was extracted with a total RNA extraction and purification kit (Bytec), and RNA integrity was detected by 1% agarose gel electrophoresis. The reverse

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transcription kit (Bectec) was used to synthesize cDNA. First, the digestion reaction solution was configured on an ice bath, making up 10 μL with gDnase 1 μL, 10 × Buffer 1 μL, total RNA 2 μL, and Rnase-free H_2O and heated at 42 ℃ for 2 min. Then, 1 μL of Stop Buffer was removed and heated at 65 ℃ for 2 min on the PCR instrument or water bath for reverse transcription. The reverse transcription reaction solution was prepared as follows: Total RNA or poly (A) RNA 0.2-2 μg, Reverse transcriptase MIX 10 μL, FQ-RT primer Mix 1 μL, and H_2O to make up 20 μL according to the following components. The reverse transcription reaction was performed on a PCR instrument under the following conditions: either at 50 ℃ for 15 min or at 72 ℃ for 2 min. The cDNA obtained can be used directly for subsequent PCR and other reactions, or frozen at −20 ℃ for later use. The first-strand cDNA synthesized by reverse transcription was used as a template for PCR amplification. The reaction program was predenatured at 94 ℃ for 5 min; 94 ℃ for 50 s and 72 ℃ for 50 s, totaling for 35 cycles; 72 ℃ 10 min. Agarose gel electrophoresis of PCR products: After the amplification was completed, 7 μL of the PCR amplification product was mixed with the loading buffer and electrophoresed on a 1.0%-1.5% agarose gel at a voltage of 150-200 V for 20-30 minutes. The sample was stained in ethidium bromide (EB) solution (10 $\mu g \cdot \mu L^{-1}$) for 10-15 minutes, and then observed with the gel imaging system.【Results】The detection results of the six viruses were all positive. Sequence analysis proved that six virus fragments had high identity with the nucleotide sequences of viruses registered in GenBank. The cherry seedlings, cherry rootstock cuttings, cherry rootstock tissue culture seedlings, and sweet cherry seedlings randomly sampled in this experiment had 86.67%, 70.00%, 0% and 76.67% virus-carrying rates. The Chinese cherry seedlings had 86.67% virus infection rate, and the proportion of PNRSV infection alone was 53.33%. The compound infection rate of PNRSV + CGRMV was the highest (6.67%). There were 2 to 3 types of virus infection and the proportions were 6.67% and 3.33%, respectively. The combined infection rates of PNRSV + ACLSV, PNRSV + LChV2, PNRSV + CGRMV + CNRMV were 3.33%; cherry necrotic rust spot virus (CNRMV) and plum necrosis were found in the samples of cuttings of cherry rootstocks. The detection rates of spotted virus (PNRSV) and cherry green ring mottle virus (CGRMV) were 36.67%, 26.67%, and 20.00%, respectively. These three viruses showed higher rates in the field of cherry cuttings. The highest percentage of compound infection was 6.67%, and there were two to three types of compound infection, the proportions were 13.34% and 6.66%, respectively. The compound infection rate of PNRSV + CNRMV + LChV2, PNRSV + CGRMV + CNRMV was 3.33%. The field virus infection rate of sweet cherry seedlings was 76.67%, 95.66% of which were infected by cherry virus A (CVA) alone, and only the combined infection of two viruses, CNRMV + A (CVA), was 3.33%; A total of 60 samples of cherry rootstock tissue culture seedlings from different production areas were not detected with 6 common viruses in cherry.【Conclusion】The field virus-carrying rates of Chinese cherry seedlings and rootstock cuttings were higher, reaching 86.67% and 70.00%, respectively. Chinese cherry seedlings *Prunus necrotic spot virus* (PNRSV) had a high infection rate and serious damage, and had a very high virus-carrying rate in Chinese cherry seedlings. Cutting propagation methods are more likely to spread and accumulate *Cherry green ring mottle virus* (CGRMV) and *Cherry necrotic rust spot virus* (CNRMV). Sweet cherry seeds cannot be recognized as virus-free by default, but were very susceptible to infection and transmission of *Cherry virus A* (CVA), which was not the best propagating material for virus-free seedlings.

Key words: Cherry；Seedlings; Virus disease; Propagating methods

近十年来随着樱桃栽培效益的提高，栽培面积逐年扩大，其病毒病的危害逐渐显现。据国外报

道，侵染大樱桃的病毒约有34种，比较常见主要有20种，我国已报道的侵染樱桃的病毒有12种[1-2]。樱桃树体感染病毒后，可阻碍树体营养生长，使树体衰弱，果实产量减少，品质下降[3-4]。张涛等[5-6]研究认为树体一旦带毒，将终身受害，无法预防与控制，培育和栽培脱毒苗是防治此病的根本途径。脱毒苗在生产中也会因栽培管理不当而再次感染病毒，有些潜隐病毒一开始并不表现症状而不易察觉[7]。吴雅琴等[8]研究发现李坏死环斑病毒通过带毒的繁育材料、种子和花粉传播；李矮缩病毒主要通过切接和芽接传播，种子和花粉也可传播。樱桃小果病毒不仅可以通过切接和芽接传播，还可通过根蘖传播，叶蝉、绿盲蝽等昆虫也是传毒媒介；苹果褪绿叶斑病毒在木本寄主上可经嫁接传染。目前樱桃苗木多以扦插、实生、组培等方式繁育，因此了解樱桃树体在不同的繁育方式下病毒的传播与积累程度对樱桃苗木病毒病的预防至关重要。

目前有关樱桃病毒病的研究多侧重于甜樱桃病毒病的发病情况调查以及病原检测[9-12]，极少有关于樱桃苗木田间带毒率的研究。目前已知的樱桃病毒中对樱桃生长发育危害较大的有7种[13]，王文文等[14]、卢美光等[15]对山东泰安、辽宁大连和北京等樱桃主产区的樱桃病毒病发生情况进行了调查和鉴定，初步明确了我国甜樱桃主产区病毒病病原主要有：李属坏死环斑病毒（PNRSV）、李矮缩病毒（PDV）、樱桃绿环斑驳病毒（CGRMV）、樱桃坏死锈斑病毒（CNRMV）、樱桃病毒A（CVA）、苹果褪绿叶病毒（ACLSV）及樱桃小果病毒2（LChV2）。李小燕等[16]研究认为用种子繁育的实生苗是不带病毒的，甚至从病树上采成的新种子也是无病毒的。本研究采用了五点取样法随机取样且样品基数较大，取中国樱桃实生苗、樱桃砧木扦插苗、甜樱桃实生苗、樱桃砧木组培苗一年生枝条，分析其所携带病毒情况，明确不同繁育方式对樱桃树体内病毒积累所产生的影响。

1　材料和方法

1.1　材料

本研究于2018—2019年进行。4种类型样品皆选自樱桃苗木基数（10万棵左右）较大育苗基地。采取对角线五点取样法[17]，每点取30株，共抽取150株，从中继续随机抽取30株调查，以保证其随机取样过程的可靠性。取中国樱桃种子实生苗（取自烟台市农科院苗木基地）样本30份；取‘兰丁2号’砧木扦插苗（取自烟台市福山区高疃镇水果帮苗木专业合作社）样本30份；取商业化樱桃砧木组培苗（取自山东省农科院和杨凌锐坤农林科技有限公司）样本30份；取甜樱桃种子实生苗（取自烟台市农科院高陵基地）样本30份。每株取相同位置、粗细一致、长度为10 cm的一年生枝条，取韧皮部放入液氮罐中冷冻，在−80 ℃冰箱内保存。提取样本总RNA，利用RT-PCR法检测6种樱桃主要病毒。

1.2　试验方法

1.2.1　总RNA提取和cDNA合成　用总RNA提取纯化试剂盒（北京百泰克生物技术有限公司）提取总RNA[18]，1%琼脂糖凝胶电泳检测RNA完整性。用反转录试剂盒（北京百泰克生物技术有限公司）合成cDNA，先在冰浴上配置消化反应液，gDNase 1 μL、10×Buffer 1 μL、Total RNA 2 μL、RNase-free H_2O 补足10 μL，在PCR仪上或者水浴上42 ℃加热2 min。然后取出加入1 μL的Stop Buffer在PCR仪上或者水浴上65 ℃加热2 min后取出以备逆转录使用。按以下组分配制反转录反应液Total RNA or poly(A)RNA 0.2~2 μg、Reverse transcriptase Mix 10 μL、FQ-RT primer Mix 1 μL、H_2O 补足20 μL。在PCR仪上按以下条件进行反转录反应：①50 ℃，15 min ②72 ℃，2 min。得到的cDNA可直接用于后续的PCR等反应，也可以−20 ℃冻存以备以后使用。

1.2.2　引物合成　引物序列参考侯义龙等[19]、刘聪利等[20]，如表1所示，由生工生物工程（上海）股份有限公司合成。

1.2.3　PCR扩增　以反转录合成的第一链cDNA为模板进行PCR扩增，25 μL反应体系。反应程序为94 ℃预变性5 min；94 ℃ 50 s，55 ℃ 50 s，72 ℃ 50 s，35个循环；72 ℃ 10 min[21-23]。PCR扩增反应体系为10×Buffer（含 Mg^{2+}）2.5 μL、2.5 mmol·L^{-1} dNTP 2.0 μL、5 U·μL^{-1} TaqDNA聚合酶0.2 μL、10 pmol·μL^{-1} 同源引物2.0 μL、10 pmol·μL^{-1} 互补引物2.0 μL、cDNA 1.0 μL、H_2O 15.3 μL，体系总量为25 μL。

PCR产物琼脂糖凝胶电泳：扩增结束后，取7 μL PCR扩增产物与上样缓冲液混匀后于1.0%~1.5%的琼脂糖凝胶以150~200 V电压电泳20~30 min。在溴化乙锭（EB）染色溶液（10 μg·μL^{-1}）中染色10~15 min，后于凝胶成像系统内观察结果[23-24]。

表1 樱桃病毒检测引物序列

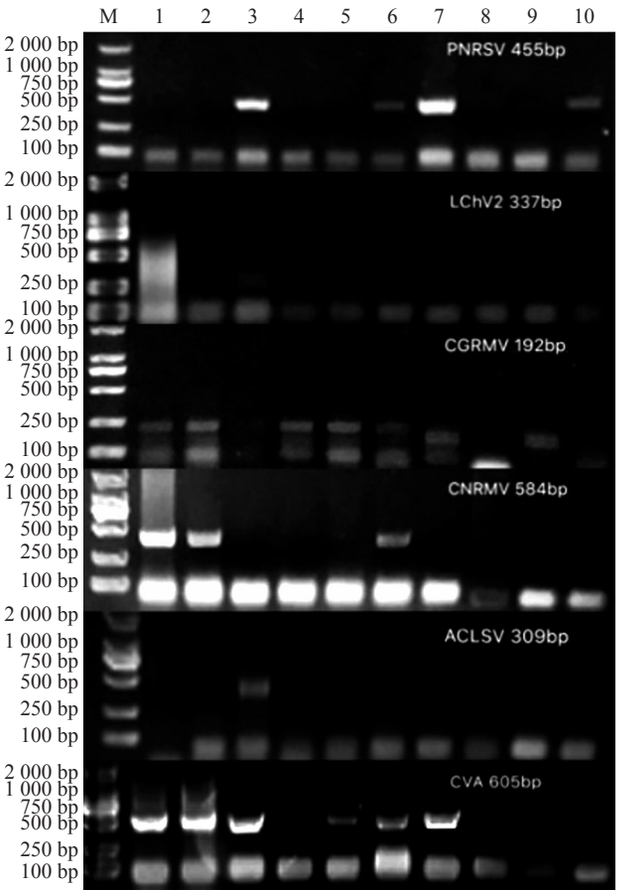
Table 1 Primer sequence cherry virus detection

病毒 Virus	引物-1 Primer-1	引物-2 Primer-2
李属坏死斑病毒 PNRSV	ACGCGCAAAAGTGTCGAAATCTAAA	TGGTCCCACTCAGAGCTCAACAAAG
苹果褪绿叶斑病毒 ACLSV	TTCATGGAAAGACAGGGGCAA	AAGTCTACAGGCTATTTATTATAAGTCTAA
樱桃绿环斑驳病毒 CGRMV	TAAACCCCTGCAATTCCACTC	CTCTAAGGAAACTGAAGGAAAA
樱桃坏死锈斑病毒 CNRMV	TCCCACCTCAAGTCCTAGCAG	TGAACTTGGCCAGTTCTGCC
樱桃小果病毒2 LChV2	TCCGAATAGTCAGTTCAAAG	AATAGCCCTCATAAATCTCC
樱桃病毒A CVA	AGCCAGAAGGTATCATGCCAG	ATGACATGCCTGCTGGGAG

2 结果与分析

2.1 樱桃病毒PCR分子鉴定电泳图

图1为本实验中6种樱桃主要病毒的部分PCR分子鉴定电泳图。



1~10 为随机取样的不同樱桃植株。

1-10 were randomly sampled from different cherry plants.

图1 樱桃主要病毒扩增产物电泳图

Fig. 1 Electrophoretogram of the main viral amplification products of cherry

2.2 中国樱桃实生苗带毒率

由图2分析得出，李属坏死斑病毒(PNRSV)和樱桃绿环斑驳病毒(CGRMV)检出率分别为70.00%和26.67%，这两种病毒中国樱桃实生苗田间携带率较高。苹果褪绿叶斑病毒(ACLSV)、樱桃坏死锈斑病毒(CNRMV)和樱桃小果病毒2(LChV2)检出率均为3.33%，李樱桃病毒A(CVA)检出率均为0%，它们在中国樱桃实生苗中的病毒检出率较低。本实验田间随机取样的30个中国樱桃实生苗样本植株带毒率为86.67%。

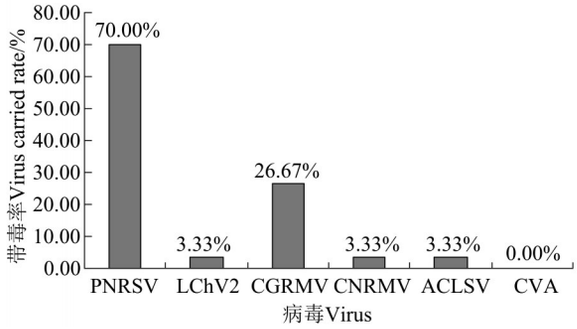


图2 中国樱桃实生苗病毒检测

Fig. 2 Chinese cherry seedling virus test

由表2得出，中国樱桃实生苗病毒侵染情况为，未被病毒侵染樱桃植株比例为13.33%，侵染一种病毒的比例为70.00%，其中单独侵染PNRSV的比例较高，为53.33%。总复合侵染比例为16.66%。PNRSV+CGRMV的复合侵染比例最高，为6.67%，复合侵染的病毒种类有2到3种，比例分别为6.67%和3.33%。PNRSV+ACLSV、PNRSV+LChV2、PNRSV+CGRMV+CNRMV的复合侵染比例均为3.33%。

2.3 扦插苗带毒率检测

由图3得出，樱桃坏死锈斑病毒(CNRMV)、李属坏死斑病毒(PNRSV)和樱桃绿环斑驳病毒(CGRMV)检出率分别为36.67%、26.67%和20.00%，这3种病毒樱桃扦插苗田间携带率较高。樱桃病毒A(CVA)和樱桃小果病毒2(LChV2)检出率分别为10.00%和3.33%，苹果褪绿叶斑病毒(ACLSV)检出率均为0%，它们在樱桃扦插苗中的病毒检出率较低。本实验田间随机取样的30个樱

表2　中国樱桃实生苗病毒复合侵染情况

Table 2　Composite infection of Chinese cherry seedling virus

复合侵染病毒数量 Number of complex infected viruses	病毒名称 Virus name					复合侵染 Compound infection	
	PNRSV	CGRMV	CNRMV	ACLSV	LChV2	样本数量 Number of samples	比例 Proportion/%
0	-	-	-	-	-	4	13.33
1	+	-	-	-	-	16	53.33
	-	+	-	-	-	5	16.67
2	+	+	-	-	-	2	6.67
	+	-	-	+	-	1	3.33
	+	-	-	-	+	1	3.33
3	+	+	+	-	-	1	3.33

注："+"表示检测到相应病毒；"-"表示未检测到相应病毒。下同。

Note: "+" indicates the corresponding virus that has been detected; "-" indicates that no virus was detected. The same below.

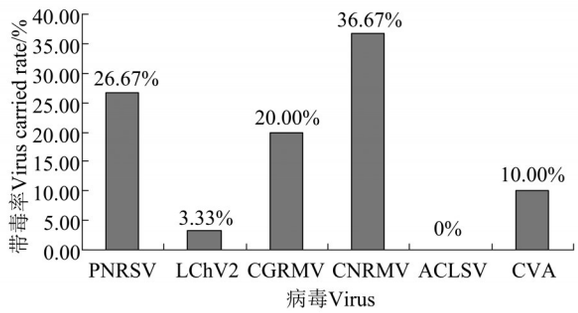


图3　樱桃砧木扦插苗病毒检测

Fig. 3　Test results of cherry rootstock cuttings

桃砧木扦插苗样本植株带毒率为70.00%。

由表3得出，樱桃砧木扦插苗病毒侵染情况为：未被病毒侵染樱桃植株的比例为30.00%；侵染一种病毒的比例为50.00%，其中单独被CNRMV侵染的比例最高，为23.33%；总复合侵染比例为20.00%，PNRSV+CNRMV和PNRSV+A(CVA)的复合侵染比例最高，均为6.67%，复合侵染的病毒种类有2到3种，比例分别为13.34%和6.66%，PNRSV+CNRMV+LChV2、PNRSV+CGRMV+CNRMV的复合侵染比例均为3.33%。

表3　樱桃砧木扦插苗病毒复合侵染情况

Table 3　Combined infection of cherry rootstock cuttings

复合侵染病毒数量 Number of complex infected viruses	病毒名称 Virus name					复合侵染 Compound infection	
	PNRSV	CGRMV	CNRMV	LChV2	CVA	样本数量 Number of samples	比例 Proportion/%
0	-	-	-	-	-	9	30.00
1	+	-	-	-	-	2	6.67
	-	+	-	-	-	5	16.67
	-	-	+	-	-	7	23.33
	-	-	-	-	+	1	3.33
2	+	-	+	-	-	2	6.67
	+	-	-	-	+	2	6.67
3	+	+	+	-	-	1	3.33
	+	-	+	+	-	1	3.33

2.4　樱桃砧木组培苗带毒率

樱桃砧木组培苗并未检测到樱桃的6种主要病毒。在第一次从山东省农科院取的30株樱桃枝条样本中未检测到所有6种主要病毒，这与预期结果不符。推测与樱桃样品的取样部位有关，之后又从杨凌锐坤农林科技有限公司取了30株樱桃的叶片，此次的检测结果依然为0%。在此之前已经检测出来樱桃实生苗和扦插苗中的病毒，且同实验室利用相同方法可检测出生长在组培瓶里的苹果组培苗中的病毒，因此排除了实验方法不可靠的可能性。

2.5　甜樱桃实生苗带毒率检测

由图4得出，樱桃病毒A（CVA）检出率为76.67%，在甜樱桃实生苗中的病毒检出率较高，樱桃坏死锈斑病毒（CNRMV)检出率为3.33%，李属坏

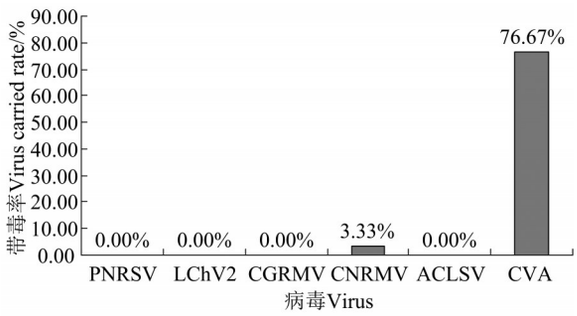


图4 甜樱桃实生苗病毒检测

Fig. 4 Sweet cherry seedling virus test

死斑病毒(PNRSV)、樱桃绿环斑驳病毒(CGRMV)、苹果褪绿叶斑病毒(ACLSV)和樱桃小果病毒2(LChV2)检出率均为0%,这几种病毒在甜樱桃实生苗中携带率较低。本实验田间随机取样的30个甜樱桃实生苗样本植株带毒率为76.67%。

由表4得出,甜樱桃实生苗病毒侵染情况为:未被病毒侵染樱桃植株的比例为23.33%;侵染一种病毒的比例为73.33%,只有CNRMV+CVA两种病毒的复合侵染,比例为3.33%。

表4 甜樱桃实生苗病毒复合侵染情况

Table 4 Combined infection of sweet cherry seedling virus

复合侵染病毒数量 Number of complex infected viruses	病毒名称 Virus name		复合侵染 Compound infection	
	CNRMV	CVA	样本数量 Number of samples	比例 Proportion/%
0	-	-	7	23.33
1	-	+	22	73.33
2	+	+	1	3.33

3 讨 论

中国樱桃是我国普遍采用的一种砧木,种子出苗率高,适应性强,但病毒病较重。本研究发现其田间带毒率为86.67%,单独侵染PNRSV的比例较高,为53.33%,覃兰英等[25]采用酶联法从樱桃上检测出4种病毒。在这些病毒中,PNRSV是为害最为严重的病毒。李坏死环斑病毒(PNRSV)是甜樱桃最严重的病毒,也是我国对外检疫病毒[8]。这一点与本研究发现的中国樱桃实生苗PNRSV侵染比例较高的情况相一致,说明李坏死坏斑病毒不仅在甜樱桃中危害严重,在中国樱桃实生苗群体中也有极高的携带率。张涛等[6]研究发现樱桃李属坏死环斑病毒病可通过嫁接、汁液摩擦等途径传播。由于中国樱桃通常被用作砧木,可能通过嫁接将PNRSV病毒传播给甜樱桃,对于甜樱桃的生产有不利影响。栽培中一般选用无病的中国樱桃作砧木,但即使植株未表现病害,其携带PNRSV病毒的风险依然很高,已嫁接好的苗木在未来几年的生产中依然可能有较高发病率,所以将中国樱桃实生苗选作砧木时应加强对PNRSV病毒的检验检疫。

培育甜樱桃实生苗是科研机构获取育种材料的常规手段,李小燕等[26]认为用种子繁育的实生苗是不带病毒的,甚至从病树上采集的新种子也是无病毒的。因此人们通常默认种子繁育的实生苗田间带毒率较低,但本研究中甜樱桃实生苗的田间带毒率为76.67%,其中95.66%为樱桃病毒A(CVA)单独侵染,带毒率非常高,复合侵染率却非常低。证实甜樱桃种子不可默认为无病毒携带,反而极易感染与传播樱桃病毒A(CVA),并非无毒苗的最佳繁育材料。这点与周登攀等[27]所发现的樱桃病毒A(CVA)的传播方式为嫁接与种传的事实相符。其不易携带除此病毒之外的其他类型病毒,因此笔者认为在甜樱桃实生苗培育过程中,需加强对樱桃病毒A(CVA)的检测。

在长期的无性繁育过程中,甜樱桃苗木感染并积累了多种病毒或类病毒[27-28],刘聪利等[29]通过对河南省樱桃病毒病的调查发现,复合侵染病毒种类为2~5种不等,侵染比例随复合侵染病毒种类增多而降低。本研究发现樱桃砧木扦插苗田间带毒率为70.00%,扦插苗病毒侵染类型有5种,其中2种病毒复合侵染的比率为13.34%,3种病毒的侵染比率为6.66%,这与刘聪利等[29]发现的复合侵染规律一致。这种情况可能是由于育苗材料良莠不齐且继代扦插快繁次数多造成,从而导致各类病毒迅速积累。扦插苗田间带毒情况中各类型病毒的侵染比率分布比较平均,只有单独侵染樱桃绿环斑驳病毒(CGRMV)和樱桃坏死锈斑病毒(CNRMV)的比例较高,分别为16.67%和23.33%。因此推测扦插繁育苗木的方式更容易传播和积累这两种病毒,或者

这两类病毒更容易通过扦插时所使用的剪刀等器械传播。因此，应建立严格的樱桃苗木育种制度，加强对扦插繁育所使用母树的病毒检验，明确规定扦插过程中所使用器械的清洁与消毒等工作。

本研究中樱桃砧木组培苗（未经脱毒处理）的检测结果与预期结果不同，从两个不同产区所取共60份样品未检测到樱桃常见的6种病毒。Hansen等[30]研究认为，植株的离体培养会破坏病毒在植株体内含量变化的规律性，连续地继代培养逐步降低ACLSV的浓度，直至最终得到脱毒苗。郭超[31]研究发现，在一个继代周期内，随培养时间的延长，病毒含量表现一定的变化趋势。其中ASGV表达量呈现先升后降再升高的趋势，而ACLSV相对含量为先升高再降低并一直维持较低水平。因此笔者推测商业化生产的组培苗经过了连续多代的继代培养而逐渐降低了樱桃植株体内的病毒含量，降低到RT-PCR检测不到的浓度，可能对植株的伤害也可能达到了最低程度。本研究中调查的是一年生的组培苗，在自然生长环境中其低浓度的病毒是否会重新变高和组培苗在长时间生产利用中的田间表现还需要进一步的检测和观察。至于具体继代培养多少次数才可以将病毒浓度降低到无害，将在今后继续深入探索。

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