

# ‘中农红’石榴组织培养和遗传转化 叶片受体材料的获得

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**摘要:**【目的】获得稳定且无菌的石榴遗传转化体系受体材料。【方法】比较不同取材时期、灭菌时间对户外石榴茎段材料的污染及褐化情况, 并进行分析。比较加入PVP和椰汁对石榴材料的影响, 筛选不同激素种类和浓度的初代培养基, 经过多次继代培养, 筛选出稳定且无菌的石榴增殖培养基, 得到稳定健壮的叶片受体材料。【结果】较利于石榴茎段初代生长的培养基为MS+1.0 mg·L<sup>-1</sup> 6-BA+0.1 mg·L<sup>-1</sup> NAA+1 g·L<sup>-1</sup> PVP。经过2次继代培养筛选出较为合适的培养基: 1.0 mg·L<sup>-1</sup> 6-BA+0.1 mg·L<sup>-1</sup> NAA+1 mg·L<sup>-1</sup> PVP+200 mg·L<sup>-1</sup> 椰汁诱导成芽最佳, 0.3 mg·L<sup>-1</sup> 6-BA+0.1 mg·L<sup>-1</sup> IBA+1 mg·L<sup>-1</sup> PVP+200 mg·L<sup>-1</sup> 椰汁诱导茎伸长最佳。在生长的石榴植株上, 能够获得翠绿、舒展健壮的‘中农红’石榴叶片。【结论】筛选得到适合‘中农红’石榴组织培养的培养基, 获得了稳定健壮且无菌的石榴试验材料, 为建立稳定的石榴遗传转化体系奠定了基础。

**关键词:** 石榴; 茎段; 组织培养; 遗传转化

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## Cultivation of the ‘Zhongnonghong’ pomegranate tissue and genetic transformation of the leaf receptor material were obtained

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**Abstract:**【Objective】In order to obtain a stable and sterile pomegranate genetic transformation system receptor material. This is the basis for conducting genetic transformation experiments, and it is also a powerful guarantee for improving reproducibility and reliability, when the proliferation buds reached 3 to 5cm, they were cut from the basal part putted into MS medium and subcultured. Add 1 mg·L<sup>-1</sup> PVP and 200 mg·L<sup>-1</sup> coconut and different concentrations of 6-BA, NAA or IBA. After two subcultures, the shoots necrosis, shoots and stem segments were observed and recorded. The rate of shoot formation was the ratio of fresh shoots to original plants on the original plants. The rate of stem formation was the ratio of stem segments grown to these shoots. A stable and sterile pomegranate proliferation medium was screened to obtain a stable and robust leaf receptor material.【Methods】The shoots of adult plants were collected from 2 to 5 knots as explants from February to March. The leaves were harvested and cutted into 1-2 stem segments, dilute the solution soak the oscillation wash 30 min, tap water rinse, placed in the clean bench sterilization, 75% alcohol surface sterilization 30 s, 0.1% HgCl<sub>2</sub> solution soaking at different times, sterile water rinse 5 times; remove the stem section inoculated with different concentrations of 6-BA, NAA or IBA

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MS, B5 or WPM on the primary medium. The culture conditions were as follows: temperature ( $24\pm 1$ ) $^{\circ}\text{C}$ , light intensity 2 500–3 000 lx, light time 16 h $\cdot\text{d}^{-1}$ . Each treatment of 20 bottles, each bottle inoculated with 3 to 4 explants, repeated 3 times. Counting the fungi, bacteria and browning from 10 d to 30 d after inoculated. When the induced axillary bud grew from 2 to 3 cm, it was cut from the basal part and transferred into the MS multiplication medium, adding 6-BA, KT, NAA and IBA, and with different concentrations. Adding 1 g $\cdot\text{L}^{-1}$  PVP anti browning, adding anti coconut juice stem tip necrosis and yellow leaves fall off. The proliferation coefficient and growth status were recorded after 30 d. Comparing the effects of PVP and coconut on the pomegranate material and screening the different medium and concentration of the first generation medium. 【Results】The medium with the first generation of pomegranate stem was MS+1.0 mg $\cdot\text{L}^{-1}$  6-BA+0.1 mg $\cdot\text{L}^{-1}$  NAA+1 mg $\cdot\text{L}^{-1}$  PVP. After two subcultures, the appropriate medium was screened: 1.0 mg $\cdot\text{L}^{-1}$  6-BA+0.1 mg $\cdot\text{L}^{-1}$  NAA+1 mg $\cdot\text{L}^{-1}$  PVP+200 mg $\cdot\text{L}^{-1}$  coconut juice induced into buds for the best, 0.3 mg $\cdot\text{L}^{-1}$  6-BA+0.1 mg $\cdot\text{L}^{-1}$  IBA+1 mg $\cdot\text{L}^{-1}$  PVP+200 mg $\cdot\text{L}^{-1}$  coconut juice for induction of stem elongation. In the growth of pomegranate plants, can get green, stretched the robust red peony leaves. 【Conclusion】The stable and robust pomegranate experimental material was obtained, which laid the foundation for the establishment of stable pomegranate genetic transformation system.

**Key words:** Pomegranate; Stem section; Tissue culture; Genetic transformation

石榴是石榴科石榴属木本植物,原产于阿富汗、伊朗等中亚地区<sup>[1]</sup>。我国从国外引种栽培石榴的历史悠久,现主要栽培地区为四川、云南、山西、河南、山东和安徽等省<sup>[2]</sup>。我国石榴资源丰富,其中软籽石榴以其甜而无渣的特点,堪称石榴贵族品种。郭俊英等<sup>[3]</sup>选育的‘中农红’软籽石榴是‘突尼斯’芽变产生的,具有皮红、果大、软籽、丰产的特点。

近年来,国内外石榴相关的研究日渐繁盛,包括育种、栽培、加工、医疗和保健等多个领域。其中石榴的组织培养工作也取得了很大进展。Shao等<sup>[4]</sup>以石榴花药为外植体成功获得四倍体植株;Murkute等<sup>[5]</sup>和Naik等<sup>[6]</sup>对石榴的幼胚进行组织培养得到愈伤组织并且成功增殖;Naik等<sup>[7]</sup>以茎段、刘广甫等<sup>[8]</sup>以茎尖为材料分别建立了石榴的再生体系;朱立武等<sup>[9]</sup>以‘玉石籽’石榴的成熟叶片为外植体,诱导出愈伤组织并分化出不定芽;Naik等<sup>[10]</sup>报道了石榴人工种子的获得。石榴组织培养比一般园艺植物困难,因为石榴内含有的酚类物质容易使培养基和材料本身发生褐变<sup>[11]</sup>,从而影响初代培养的成活率;在继代培养过程中易发生叶片干枯脱落<sup>[12]</sup>现象,从而影响成活率和增殖系数;芽在继代过程中易死亡;不定芽分化难使得增殖系数不理想<sup>[13]</sup>。随着组织培养技术越来越多地向遗传转化<sup>[14]</sup>、生物技术育种<sup>[15]</sup>和转基因工程方向发展<sup>[16-18]</sup>,笔者以‘中农红’石榴带芽茎段为外植体材料,旨在解决石榴组培中常见的问题,为建立稳定高效的遗传转化体系提供关键技术。

## 1 材料和方法

### 1.1 试验材料

植物材料:以中国农业科学院郑州果树研究所苗圃基地的‘中农红’石榴为材料,于2017年2—3月采集成年植株枝条上长至2~5个节的带芽茎段为外植体材料。

试剂:聚乙烯吡咯烷酮[Polyvinylpyrrolidone, PVP (40 000)]、琼脂粉(3 000~9 000)和激素都是购自Solarbio公司,蔗糖购自天津市致远试剂有限公司,椰汁为新鲜椰子汁,其他MS培养基药品为国药生产。

### 1.2 外植体的消毒与初代培养

取新萌发的幼嫩枝条,去掉叶片,剪成带1~2个芽的茎段,用洗洁精稀溶液浸泡振荡洗涤30 min,自来水冲洗干净后,置于超净工作台上灭菌。75%酒精表面杀菌30 s,0.1%的 $\text{HgCl}_2$ 溶液浸泡振荡不同时间,无菌水冲洗5遍;取出茎段接种于加有不同浓度6-BA、NAA或IBA的MS、B5或WPM初代培养基上。培养条件为:温度( $24\pm 1$ ) $^{\circ}\text{C}$ ,光照强度2 500~3 000 lx,光照时间16 h $\cdot\text{d}^{-1}$ ,下同。每个处理20瓶,每瓶接种3~4个外植体,3次重复。10~30 d内统计真菌、细菌污染情况和褐化情况。

### 1.3 初代培养

当诱导出的腋芽长至2~3 cm时,从基部切下后转入MS增殖培养基中,添加6-BA、KT、NAA和IBA,并设不同浓度。加入1 g $\cdot\text{L}^{-1}$  PVP防褐变,加入

椰汁防茎尖坏死和叶片发黄脱落。经过2次继代培养后观察记录增殖系数和长势。本研究中,石榴的增殖系数以其节位上有腋芽萌发,并且长成茎段(大于1 cm)为标准。

#### 1.4 多次继代培养

增殖芽长到3~5 cm时,从基部切下,转入MS培养基中再继代培养。加入 $1\text{ g}\cdot\text{L}^{-1}$  PVP、 $200\text{ mL}\cdot\text{L}^{-1}$  椰汁和不同浓度6-BA、NAA或IBA。30 d后观察并记录其茎尖坏死、成芽和长成茎段的情况。成芽率是在原有植株上新生的芽与原植株的比值,成茎率是这些芽生长成的茎段与原植株的比值。

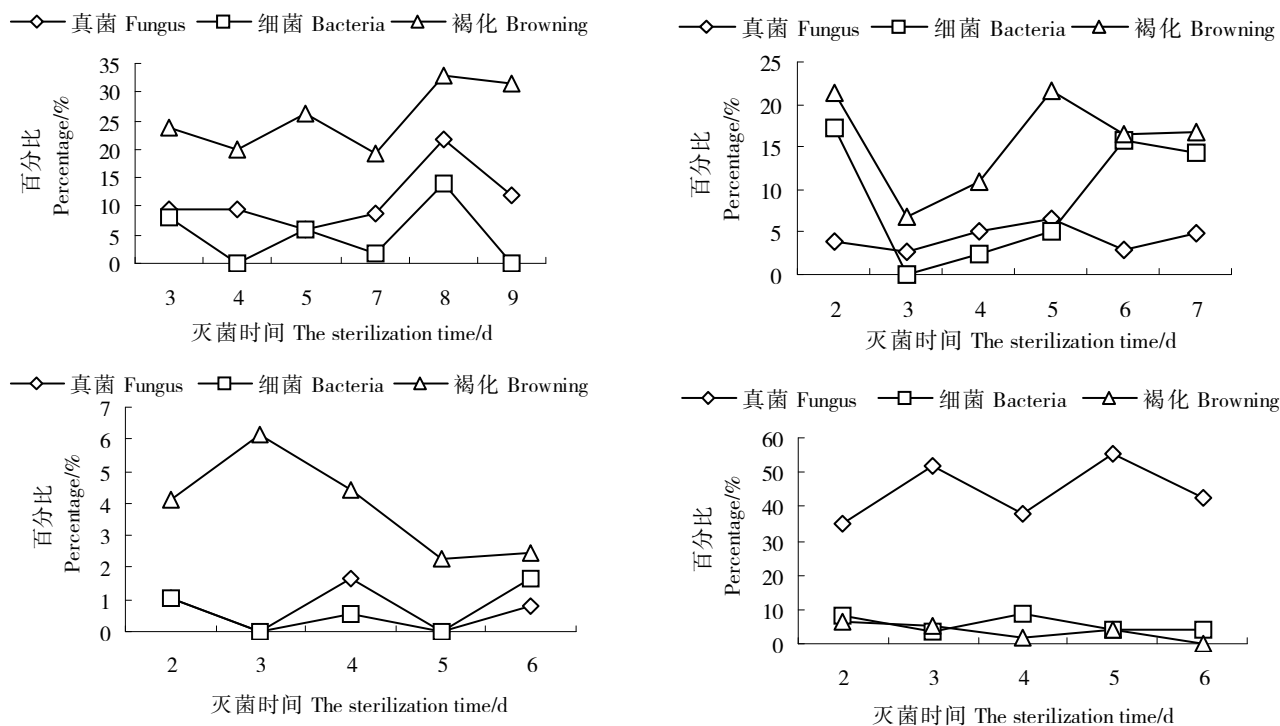
#### 1.5 数据处理

试验数据采用SPSS统计软件按照最小显著差数法(LSD法)进行多重比较。

## 2 结果与分析

### 2.1 不同灭菌时间对茎段外植体灭菌效果的影响和比较

灭菌后的外植体移至培养基上,叶片3~7 d后会出现褐变和污染的情况,而茎段1~3 d出现褐变,3~7 d出现污染。从图1可以看出,随着采样时期的推迟,在以上灭菌条件下,植株褐化死亡率逐渐降低。但是植株的染菌率越来越高,尤其是真菌。细菌染菌率一般能够控制在0.2%以内。综上所述,温室大棚内植株材料控制染菌率的关键是控制真菌污染率,而升汞消毒方法对真菌孢子的杀灭效果不佳,因此灭菌效果跟外植体生长的外界环境有极大的关系,具有不稳定性。



A. 2月28日; B. 3月4日; C. 3月15日; D. 3月29日。

A. Feb. 28; B. Mar. 4; C. Mar. 15; D. Mar. 29.

图1 不同灭菌时间对茎段外植体灭菌效果的影响

Fig. 1 Effect of different sterilization time on sterilization of stem explants

### 2.2 不同激素对比对茎段初代培养的影响

如表1所示,激素6-BA的诱导效果较KT和TDZ好,处理22的茎段基部直接脱分化为愈伤,原有茎尖坏死,叶片脱落。

随着6-BA激素用量的增加,腋芽成茎率有所增加,同时茎也逐渐健壮,处理10和处理11的成茎率均很高,处理6的成芽数最多,为5.2,但6-BA用量太多使腋芽丛生现象严重,难以分割并加重幼苗

的玻璃化,以 $1.0\text{ mg}\cdot\text{L}^{-1}$ 为最佳。

6-BA用量相同时,NAA比IBA的诱导效果好且成芽质量高,表现为无玻璃化苗,无徒长现象,节间长度适当,叶片数多;IBA的用量以 $0.1\text{ mg}\cdot\text{L}^{-1}$ 为最佳。

综合来看,石榴带芽茎段的最佳初代培养基为MS+ $1.0\text{ mg}\cdot\text{L}^{-1}$  6-BA+ $0.1\text{ mg}\cdot\text{L}^{-1}$  NAA+ $1\text{ mg}\cdot\text{L}^{-1}$  PVP,成茎段率为198.56%,诱导芽长势良好(图2)。

表 1 不同激素对比对茎段初代培养的影响

Table 1 Effects of different hormone combinations on the primary culture of stems

处理 Treatment	基本培养基 Basal medium	$\rho(6\text{-BA})/$ ( $\text{mg}\cdot\text{L}^{-1}$ )	$\rho(\text{KT})/$ ( $\text{mg}\cdot\text{L}^{-1}$ )	$\rho(\text{TDZ})/$ ( $\text{mg}\cdot\text{L}^{-1}$ )	$\rho(\text{IBA})/$ ( $\text{mg}\cdot\text{L}^{-1}$ )	$\rho(\text{NAA})/$ ( $\text{mg}\cdot\text{L}^{-1}$ )	成茎率 Percentage of stem formation/%	成茎质量 Stem quality
1	MS	0.60	-	-	0.10	-	53.6 e	++
2	MS	0.80	-	-	0.10	-	42.5 e	++
3	MS	1.00	-	-	0.10	-	53.2 e	++
4	MS	1.00	-	-	0.30	-	134.4 b	+++
5	MS	1.00	-	-	0.50	-	168.3 a	++++
6	MS	1.20	-	-	0.10	-	130.6 b	++++
7	MS	1.50	-	-	0.10	-	88.9 c	+++
8	MS	0.60	-	-	-	0.10	58.9 d	++
9	MS	0.80	-	-	-	0.10	116.7 b	+++
10	MS	1.00	-	-	-	0.05	173.7 a	++++
11	MS	1.00	-	-	-	0.10	189.6 a	++++
12	MS	1.00	-	-	-	0.20	108.8 b	+++
13	MS	1.20	-	-	-	0.10	53.3 e	++
14	MS	1.50	-	-	-	0.10	108.3 b	++
15	MS	-	0.50	-	-	0.10	106.3 b	+++
16	MS	-	1.00	-	-	0.10	138.9 b	+++
17	MS	-	1.50	-	-	0.10	88.9 c	+++
18	MS	-	2.00	-	-	0.10	67.8 d	++
19	MS	-	-	0.00	-	0.10	73.8 d	++
20	MS	-	-	0.01	-	0.10	31.1 e	+
21	MS	-	-	0.10	-	0.10	15.1 f	+
22	MS	-	-	0.20	-	0.10	0.0 f	-
23	B5	1.00	-	-	-	0.10	159.9 a	+++
24	MS	-	-	-	-	-	100.0 c	++

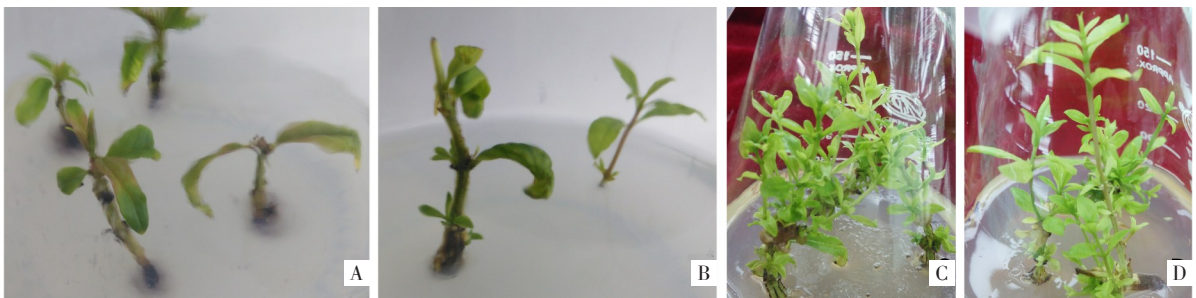
注:植株成茎质量分为5级,“-”表示没有诱导成芽;“+”表示诱导芽有黄化现象,芽丛生且难以分割继代,叶片小而密集;“++”表示诱导芽轻度黄化、徒长且叶片数少,成芽数不理想;“+++”表示诱导芽无黄化现象,但成芽数较少;“++++”表示诱导芽无黄化现象,成芽数最恰当且适合分割,节间长度和叶片数最合适,最适宜继代培养。表中数字后小写英文字母分别表示在  $P < 0.05$  上差异显著(LSD 测验)。下同。

Note: The quality of the stem is divided into 5 levels, “-” indicates no induced into bud; “+” indicates bud induction etiolation, bud clumps and difficult to split transgenerational, leaf blade small, dense; “++” indicates induced bud light yellow, moderate growth and leaf number less, the bud number is not ideal; “+++” indicates induced bud no yellowing phenomenon, but a bud number is less; “++++” indicates induced shoots without blanching, bud for the most appropriate and suitable for segmentation, internode length and leaf number of the most suitable, most suitable for successive transfer culture. The different small letters indicate the significant difference at  $P < 0.05$  LSR test. The same below.

### 2.3 PVP和椰汁对石榴组织培养的影响

由图2可知,没有添加PVP的石榴初代培养基上幼苗茎基部出现褐化现象。同时没有加椰汁的石

榴苗从初代培养时已经出现叶片发红或者发黄现象,添加了 $200\text{ mL}\cdot\text{L}^{-1}$ 椰汁后,增殖芽的干枯落叶和茎尖坏死现象得到一定程度的改善。



A. 未加PVP,无椰汁;B. 加PVP,无椰汁;C. 处理5,加PVP,加椰汁;D. 处理12,加PVP,加椰汁。

A. No PVP, no coconut juice; B. PVP, no coconut juice; C. Treatment 5, PVP and coconut juice; D. Treatment 12, PVP and coconut juice.

图 2 激素、PVP 和椰汁对石榴组织培养的影响

Fig. 2 Effect of hormone, PVP and coconut juice on tissue culture of pomegranate

### 2.4 不同培养基对多次继代培养的影响

经过2次继代增殖,由表2可以看出,茎尖坏死率均很高,叶片枯黄脱落,在茎段的基部出现新芽,

部分芽已经生长成茎段。通过比较发现,处理13和 处理1的成芽率较高,处理13的茎尖坏死率也很高。本研究得出: $1.0\text{ mg}\cdot\text{L}^{-1}$  6-BA+ $0.1\text{ mg}\cdot\text{L}^{-1}$  NAA

表 2 不同培养基对多次继代培养的影响

Table 2 Effects of different medium on repeated subculture

处理 Treatment	基本培养基 Basal medium	$\rho(6\text{-BA})/$ ( $\text{mg}\cdot\text{L}^{-1}$ )	$\rho(\text{KT})/$ ( $\text{mg}\cdot\text{L}^{-1}$ )	$\rho(\text{IBA})/$ ( $\text{mg}\cdot\text{L}^{-1}$ )	$\rho(\text{NAA})/$ ( $\text{mg}\cdot\text{L}^{-1}$ )	茎尖坏死率 Shoot tip necrosis rate/%	成芽率 Bud formation rate/%	成茎率 Percentage of stem formation/%	成茎质量 Stem quality
1	MS	0.30	-	0.10	-	75.0 a	141.7 a	83.3 a	++
2	MS	0.50	-	0.10	-	105.6 a	61.1 a	44.4 b	++
3	MS	1.00	-	0.10	-	31.1 b	53.5 b	44.9 b	++
4	MS	-	0.50	0.10	-	36.7 b	15.0 b	15.0 b	+
5	MS	0.30	0.50	0.10	-	25.0 b	42.3 b	30.6 b	+
6	MS	0.50	0.50	0.10	-	69.0 a	52.9 b	42.5 b	++
7	MS	1.00	0.50	0.10	-	116.7 a	87.5 a	45.8 b	++
8	MS	0.30	1.00	0.10	-	42.9 b	56.9 a	60.1 a	+++
9	MS	0.50	1.00	0.10	-	57.8 b	28.9 b	28.9 b	++
10	MS	1.00	1.00	0.10	-	70.8 a	29.2 b	12.5 b	+
11	MS	0.30	-	-	0.10	111.1 a	122.2 a	111.1 a	++++
12	MS	0.50	-	-	0.10	10.3 b	95.2 a	94.4 a	+++
13	MS	1.00	-	-	0.10	122.2 a	161.1 a	127.8 a	+++
14	MS	0.30	0.50	-	0.10	81.1 a	161.1 a	37.8 b	++
15	MS	0.50	0.50	-	0.10	54.8 b	103.2 a	54.0 a	++
16	MS	1.00	0.50	-	0.10	31.9 b	30.6 b	23.6 b	+
17	MS	0.30	1.00	-	0.10	69.4 a	34.7 b	19.4 b	+
18	MS	0.50	1.00	-	0.10	64.2 a	15.0 b	10.8 b	+
19	MS	1.00	1.00	-	0.10	33.3 b	66.7 a	27.8 b	++

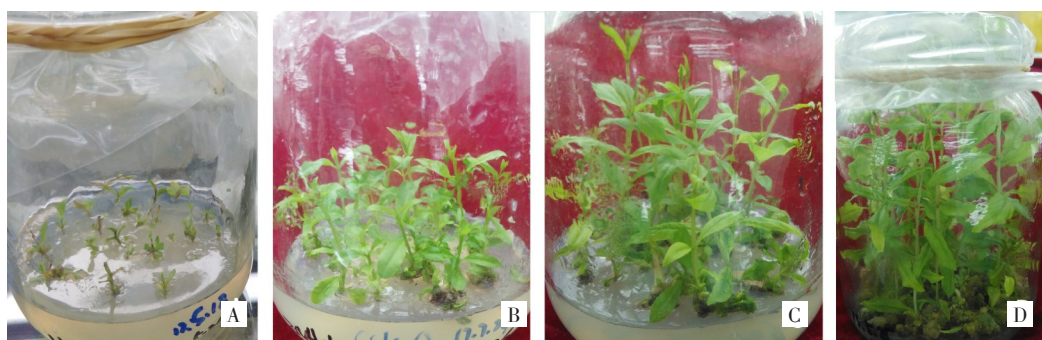
注: 植株成茎质量分为 4 级, “+”表示株高和茎粗很弱, 叶面积小, 叶片数少, 叶色淡绿甚至枯黄; “++”表示株高和茎粗细弱, 叶面积适中, 叶片数较多, 叶色淡绿甚至枯黄; “+++”表示株高和茎粗较壮, 叶片伸展, 叶面积大, 叶片数较多, 叶色鲜绿甚至枯黄。“++++”表示株高和茎粗理想, 叶片伸展, 叶面积大, 叶片数很多, 叶色鲜绿, 无枯黄。

Note: Stem quality is divided into 4 levels, “+” indicates that the plant height and stem are very weak, the leaf area is small, the leaves are few, and the leaves are pale green and even yellow. “++” indicates the thickness of the stalk and height, the leaf area is moderate, the leaf number is more, the leaf color is light green and even yellow; “+++” indicates that the plant height and stem are strong, the blade stretches, the leaf area is large, the leaves number is more, the leaf color is bright green and even yellow. “++++” indicates the height of the plant and the coarse ideal of the stem, the blade stretches, the leaf area is large, the leaves are numerous, the leaves are bright green, and no yellow.

诱导成芽最佳,  $0.3 \text{ mg}\cdot\text{L}^{-1}$  6-BA+ $0.1 \text{ mg}\cdot\text{L}^{-1}$  IBA 诱导茎伸长最佳。

## 2.5 优质叶片受体材料的获得

由图 3 可以看出, 石榴先在诱导成芽培养基上



A. 刚继代接入; B. 继代 12 d; C. 继代 15 d; D. 继代 30 d。

A. Just inherited access; B. Subculture for 12 days; C. Subculture for 15 days; D. Subculture for 30 days.

图 3 优质叶片受体材料的获得

Fig. 3 Acquisition of high quality leaf receptor material

生长, 然后继代接入诱导茎伸长的培养基上, 石榴的生长状态极好, 15~30 d 的石榴叶片可作为石榴遗传转化的稳定受体材料。

## 3 讨 论

石榴带菌外植体材料灭菌时间与其生长状态有

很大的关系。石榴叶片外植体经过70%乙醇30 s, 0.1%的HgCl<sub>2</sub>灭菌,张晓申等<sup>[19]</sup>认为最佳灭菌时间为2.5 min,陈延惠等<sup>[20]</sup>认为4~5 min最佳。这些结果说明不同时期、不同的外植体生长状态以及不同的外界菌体污染情况,最佳灭菌时间是不同的。因此直接采用带菌外植体作为遗传转化受体材料是不可重复的,同时对材料也是有损害的,王菲等<sup>[21]</sup>认为成熟度、灭菌时间会影响脱分化和再分化能力,同时随着继代次数增多,无菌苗内部分裂素水平升高,更有利于芽再分化。

石榴含有很多酚类物质,因此褐变是石榴组织培养中普遍存在的现象。王雪婧<sup>[22]</sup>采用培养基中加入100 mg·L<sup>-1</sup> 维生素C的方法,陈海燕<sup>[23]</sup>采用降温和初期弱光培养的方法来降低褐化率;黄剑华等<sup>[12]</sup>采用前期弱光培养、后期逐渐转入正常光照培养的方法来减缓软籽石榴组培中的褐变现象;王菲等<sup>[24]</sup>将灭菌后的外植体浸泡在4‰的PVP溶液中10 min,在浸泡中酚类物质被稀释并被PVP吸收,大大减轻了酚类物质对外植体的毒害作用。笔者在培养基中加入PVP也能缓解软籽石榴的褐化现象。

叶枯脱落和茎尖坏死现象是影响石榴增殖的制约性因素,这在王菲等<sup>[24-25]</sup>、王雪婧<sup>[22]</sup>和陈海燕<sup>[23]</sup>的研究中都有报道。王菲等<sup>[24-25]</sup>通过封口膜的透气性差异试验发现瓶内有害气体的累积与茎尖坏死关系不大。椰汁的加入抑制了石榴组培苗的落叶并促进正常生长,这与王菲等<sup>[24-25]</sup>、陈海燕<sup>[23]</sup>和黄剑华等<sup>[12]</sup>在石榴上的研究结果一致。陈平华等<sup>[26]</sup>和Perán-Quesada等<sup>[27]</sup>认为椰汁能够增加促进细胞分裂的物质,但Pacheco等<sup>[28]</sup>、Peixe等<sup>[29]</sup>、Ismail等<sup>[30]</sup>和Santos-Hernández等<sup>[31]</sup>认为椰汁为组培苗生长提供了某些必需的营养成分。

椰汁可以有效地抑制茎尖坏死和叶片脱落,但是相同的培养条件,经过多次继代培养,石榴组培苗依然会枯萎死亡。获得健壮、翠绿、舒展的石榴叶片是建立稳定的遗传转化体系的关键,笔者发现石榴茎尖适应的激素浓度远远低于以往文献报道<sup>[10,16]</sup>,但是该浓度又不能达到增殖培养的目的,根据不同石榴部位和不同的继代周期选择不同的培养基,能够有效地解决石榴组培苗稳定培养的问题。

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