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Efficient Induction of Papaya Embryogenic Callus and Plant Regeneration

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Abstract Papaya (*Carica papaya* L.) has high medicinal and production value, and it is of great significance to carry out efficient induction and plant regeneration of embryogenic callus of papaya for its industrial development. In this study, Zhongbai, the main papaya cultivar from Hainan was used as explants to explore the effects of different hormones and their additive combinations on embryogenic callus induction and plant regeneration. The results showed that the most suitable medium for papaya callus induction was K5: 1/2 MS+5 mg/L 2, 4-D+0.5 mg/L KT+3% sucrose+3.6 g/L phypagel. The most suitable medium for papaya embryogenic callus induction was M13: 1/2 MS+5 mg/L 2,4-D+0.6 mg/ L-proline+3% sucrose+3.6 g/L phypagel. And the most suitable medium for papaya bud differentiation was Ci: 1/2 MS+0.2 mg/L 6-BA, 0.2 mg/L NAA+3% sucrose+3.6 g/L phypagel. The best method for rooting of papaya was treatment with 100 mg/L rooting powder and soil plantation. This 'two-steps' embryogenic callus induction system we created can significantly shorten the growth cycle of embryogenic callus. Furthermore, the system has a certain broad spectrum in different varieties of papaya, this study provides a solid foundation for further improving the efficiency of genetic transformation of papaya.

Keywords Papaya; Hypocotyl; Callus; Regeneration

Papaya (*Carica Papaya* L.), also known as marigold fruit, is a tropical and subtropical evergreen large perennial herb, mainly planted in Guangdong, Hainan, Guangxi, Yunnan, Fujian, Taiwan and other areas, is one of the four famous fruits in the south of the Lingnan. Papaya has a high nutritional value, ranking first among 34 fruits in terms of its nutritional value assessed by 8 essential vitamins and minerals for human body (Malabadi et al., 2011). Papaya also has great medicinal and production value. Papaya is the main food recommended to solve vitamin A deficiency (Chandrika et al., 2003), and papaya enzyme is widely used in the food industry and medical field (Bhattacharya and Khuspe, 2001).

The planting of papaya is mainly dominated by seedling growth, but the economic benefits of conventional planting are very low due to the small number of self-rearing varieties in Chinese papaya, the complexity of features of flowers, the vulnerability to virus infection in the growth process, and the difficulty of traditional planting to maintain the good traits of the mother parent (Zhou et al., 2010). Domestic scientists have experimented with different planting methods, including introduction and domestication, cross breeding and mutagenesis breeding, but the results are not satisfactory. Through years of practical experience, it is found that genetic engineering improvement technology is an effective and feasible technical means (Lyu et al., 2019). Therefore, the establishment of callus induction and regeneration technology is an important basis for improving germplasm characteristics of papaya by genetic engineering.

Scientists at home and abroad have reported callus induction using ovule, cotyledon (Zeng et al., 2003), leaf (Zhu and Zhang, 2001), root and stem (Huang et al., 1996) as starting explants of papaya, and conducted in-depth studies on somatic embryos and regenerated plants induced by callus. The induction efficiency of somatic embryo of papaya varied with the variety, the age of the explants used and the genotype of papaya. At the same time, the types and concentrations of hormones and other additives in the medium play an important and obvious role in organ differentiation and embryoid formation. For example, the addition of CH, proline (L-Pro), mannitol, $AgNO_3$,

Ca^2 and other substances in the medium have certain effects on induction and differentiation of embryogenic callus (Ji et al., 2002).

Although there have been many reports on callus induction by different explants, there are common problems in all of them. The induction cycle is long and the induction system is subject to great differences in varieties. The callus induction and differentiation system published so far is only applicable to the tested varieties. Research on embryogenic callus induction and differentiation of papaya is the premise of genetic engineering. Therefore, screening out callus induction system suitable for multiple varieties and shortening the time of embryogenic callus maturation and regeneration has important guiding significance for improving the efficiency of genetic transformation of papaya.

The 'Zhongbai' variety of papaya is a hybrid variety with unclear background. 'Zhongbai' is widely cultivated in Hainan for its good quality. In this study, the hypocotyl of Hainan papaya 'Zhongbai' was used as the main material, and the formation of embryogenic callus was divided into four stages: explant expansion stage (Stage I), primary callus formation stage (Stage II), embryogenic callus formation stage (Stage III) and embryogenic callus propagation stage (Stage IV). Finally, the optimum medium formula for callus induction and embryogenic callus induction was screened, and the medium formula for somatic embryo regeneration and rooting was screened. Embryogenic callus formation and regeneration period were significantly shortened, and embryogenic callus induction system was suitable for 7 varieties tested. This provides an important basis for the further efficient development of genetic transformation of papaya and has a broad prospect of development and application in the actual production and scientific research of papaya.

1 Results and Analysis

1.1 Acquisition of explants and induction of hypocotyl callus

Previous studies have reported that cotyledon, young embryo, leaf and hypocotyl are generally selected as explants for callus induction in tissue culture of papaya. However, cotyledon and leaf have low callus induction ability, and the acquisition period of young embryo materials is relatively long and embryo removal is time-consuming. In this study, hypocotyl cultured under darkness was used as explants. The hypocotyl cultured under darkness was more than twice as long as that cultured under light, and more hypocotyl segments were obtained as explants, and the hypocotyl segment was younger and more suitable for callus induction (Figure 1). This method greatly increased the number of explants.

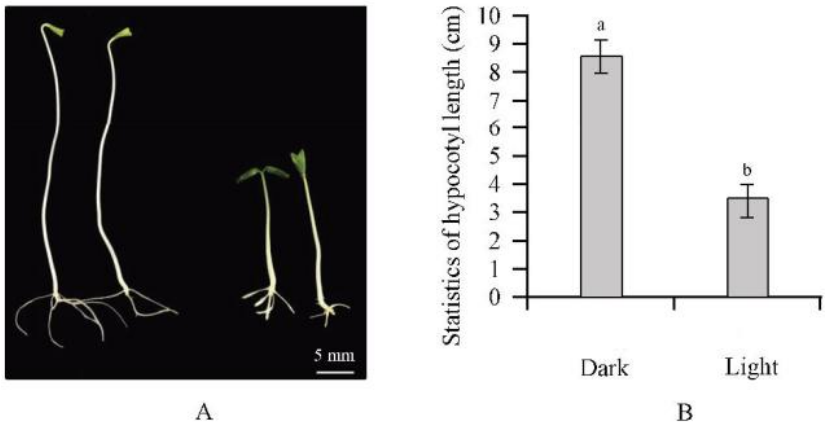

Figure 1 Hypocotyls of papaya grown under light and dark

Note: A: Hypocotyls of papaya grown under light and dark for 8 days; B: Statistics of hypocotyl length. Scale=5 cm; Different lowercase letters in the table indicate significant differences at 0.05 level

After the young hypocotyl segment was inoculated on different callus induction medium (Table 1), it could be found that the explants began to expand into callus with different shapes after 4~5 days under the effect of KT, while the explants did not expand significantly on the medium without KT. We called this stage explant expansion (Stage I) (Figure 2; Figure 3C). The callus changed from white translucent to yellow mass after the explants were cultured for 20 to 30 days. With the increase of KT concentration (Figure 2), hypocotyl segment enlargement gradually increased, and the callus became looser. As the concentration of 2,4-D decreased, the callus formation rate gradually decreased. When the concentration of 2,4-D was lower than 5 mg/L, the callus began to sprout, indicating that the optimal concentration of 2,4-D for callus induction should not be lower than 5 mg/L. After about 30 days of culture on induction medium, the primary callus formed. We defined this stage as callus formation stage (Stage II). Both callus expansion stage and formation stage were placed on the same medium. The statistical results showed (Table 1) that different content combinations of 2,4-D and KT had different callus induction effects, and K5 medium had the highest induction rate. The difference with other medium was significant. The optimal medium for callus induction was K5: 1/2 MS+5 mg/L 2, 4-D+0.5 mg/L KT+3% sucrose +3.6 g/L plant gel.

Table 1 Effect of 2, 4-D and KT on compact on callus induction from hypocotyl

Medium number	Basic medium 1/2MS	2,4-D concentration (mg/L)	KT concentration (mg/L)	Sucrose concentration (g/L)	Number of inoculated hypocotyles	Callus induction rate (%)
K1	1/2 MS	10	_	30	60	78.33±3.76^{c}
K2	1/2 MS	10	0.5	30	60	87.33±4.08^{a}
K3	1/2 MS	10	0.1	30	60	85.33±3.39^{a}
K4	1/2 MS	5	_	30	60	72.43±4.48^{d}
K5	1/2 MS	5	0.5	30	60	90.12±5.18^{a}
K6	1/2 MS	5	0.1	30	60	83.11±5^{b}
K7	1/2 MS	2	_	30	60	50±4^{e}
K8	1/2 MS	2	0.5	30	60	43±4.13^{f}
K9	1/2 MS	2	0.1	30	60	40.67±6.03^{f}

Note: Different lowercase letters in the same column in the table indicate significant differences at 0.05 level

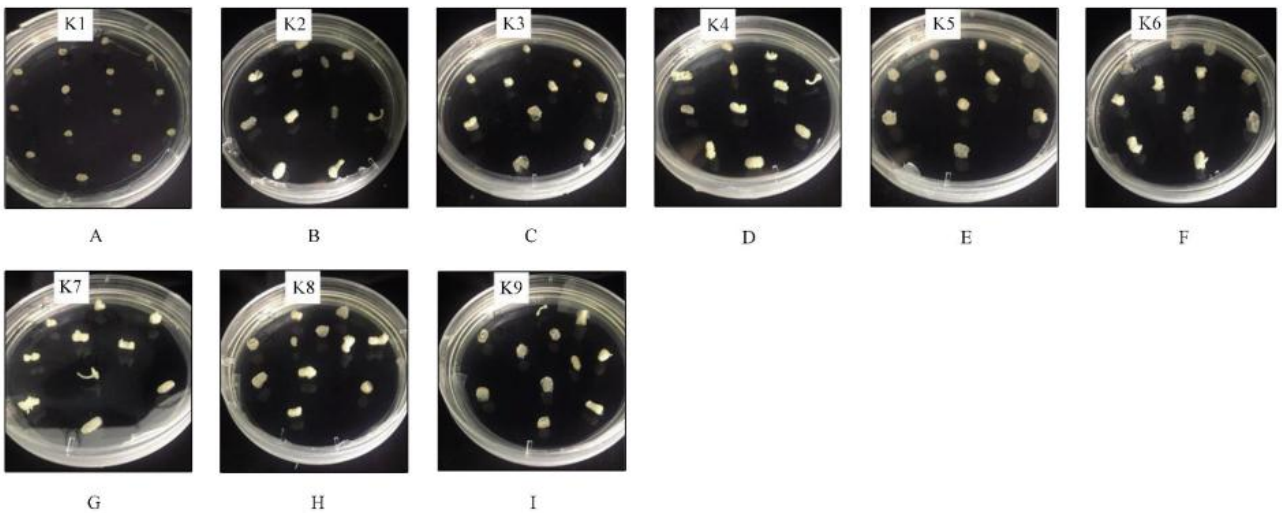

Figure 2 Status of explants on different induction media

Note: A~I: The state of callus formed after explants grown on K1~K9 medium for 12 days

1.2 Effect of hormone combination on embryogenic callus formation

After 60 days of callus subculture, callus significantly expanded and some callus changed from yellow to yellowish brown. However, the efficiency of embryogenic callus induction on K1-K9 medium was very low, so it was necessary to select the most suitable embryogenic callus induction medium. Callus cultured on K5 for 30 days were inoculated on embryogenic callus induction medium with different hormone combinations. After 3

months of culture, pale yellow embryogenic callus with spherical particles on the surface could be observed by naked eye (Figure 3D). As can be seen from the table (Table 2): (1) 2,4-D played a major role in the induction of embryogenic callus from callus, and KT was not conducive to the induction of embryogenic callus; (2) Vitamin significantly promoted embryogenic callus induction, but hydrolyzed casein, glutamine and glycine did not significantly promote embryogenic callus induction. (3) L-Pro promoted the growth of embryogenic callus of papaya, increased the rate of embryogenic callus formation, and shortened the time of somatic embryo formation of papaya by about 2 months; (4) The optimal medium for callus embryo induction was M13: 1/2 MS+5 mg/L 2, 4-D+0.6 mg/L L-Pro+25 mg/L vitamin+3% sucrose +3.6 g/L plant gel.

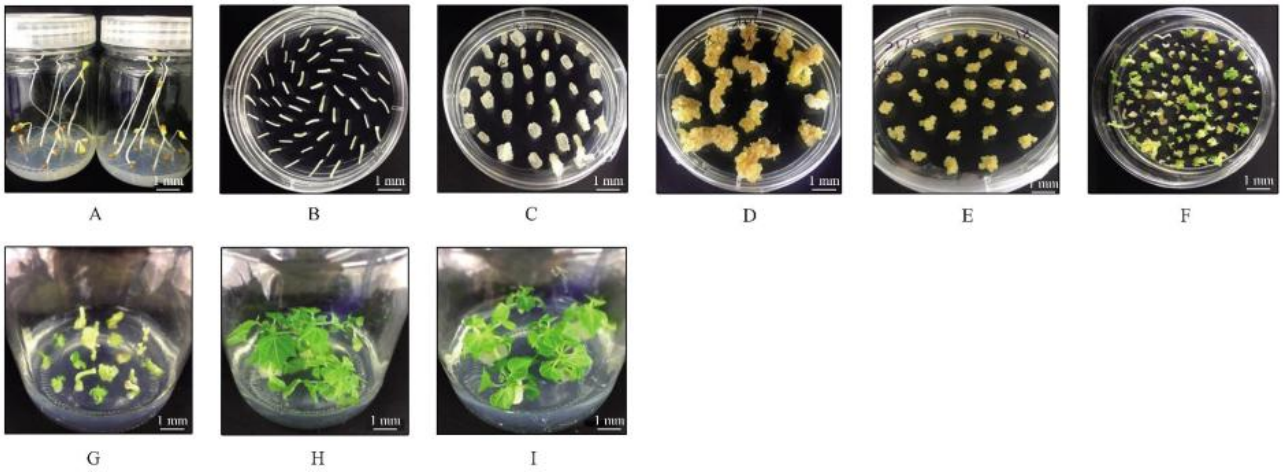

Figure 3 Embryogenic callus induction and somatic embryo regeneration in papaya

Note: A: Hypocotyl explants were grown on 1/2MS medium for 7 days; B: The hypocotyl segment were placed on M5 medium; C: Hypocotyl explants were grown on M5 medium for 5 days; D: The callus began to grow embryogenic callus after 60 days on M13 medium; E: Embryogenic callus was expanded on Ci medium for 30 days; F-G: Somatic embryos began to grow cotyledon after 60 days of growth on MBN2 medium; H-I: Somatic embryos grow into complete plants; Scale = 1cm

When the hypocotyl segment was directly cultured on M1 medium for 3 months, it was found that the callus formed by the hypocotyl was white and transparent, without swelling or long callus. Somatic embryogenesis was evident in the callus after hypocotyl was cultured on K5 medium for 1 month and then transferred to M13 for 2 months (Figure 4). These results indicated that only 2,4-D was added during explants' expansion stage (Stage I) and primary callus formation, and the time of callus formation was very slow, while K5+M13 medium could effectively induce embryogenic callus formation.

The embryogenic callus on the surface of the callus was selected and placed on Ci medium for propagation. This Stage is called Stage IV of embryogenic callus propagation. In our laboratory, the best medium for embryogenic callus proliferation was Ci: 1/2 MS+2, 4-D 10mg/L+7% sucrose + 3.6g /L plant gel, pH 6.0. High concentration of 2,4-D and sucrose can significantly improve the propagation rate of embryogenic callus, which can multiply 7-10 times within one month (Figure 3E).

1.3 Effect of K5, M13 embryogenic callus induction medium on different papaya cultivars

In order to further verify the efficiency of callus induction system of ‘Zhongbai’ papaya screened by our laboratory, we also tried callus induction effect of other varieties of papaya, including ‘Hongfei’, ‘Hongling 1’, ‘Hongling 2’, ‘Zhonghuang’, ‘AU9’, ‘SunUp’ (Table 3). It can be seen that the somatic embryo induction efficiency of different varieties is more than 50%, and the induction rate of ‘Zhonghuang’ is up to 85%. The induction rate of SunUp was 73%, and ‘Zhonghuang’ and ‘AU9’ explants could start to develop embryogenic callus after 9 weeks of induction, indicating that the callus induction rate of different varieties was significantly different. The results showed that the two-step system of embryogenic callus induced by this method had obvious advantages and was universal to different varieties of papaya.

Table 2 Effect of 2,4-D, KT and different nutrients on compact on callus induction from hypocotyl

Medium number	1	2	3	4	5	6	7	Sucrose concentration (g/L)	Number of inoculated callus 1	Rate of embryogenic induction (%)	Formation time of Embryogenic callus (weeks)	Feature of embryogenic callus
M1	1/2 MS	10	-	1	25	2	-	30	60	63.27±4.95^{c}	20	Yellow, granular
M2	1/2 MS	10	0.1	1	25	2	-	30	60	58.67±3.06^{d}	22	Light yellow, white, granular
M3	1/2 MS	10	0.5	1	25	2	-	30	60	52.37±9.22^{e}	22	Light yellow, white, granular, water stained
M4	1/2 MS	5	-	1	25	2	-	30	60	46.83±4.65^{f}	18	Yellow, grain, loose
M5	1/2 MS	5	0.1	1	25	2	-	30	60	37.73±7.18^{g}	19	Light yellow, loose
M6	1/2 MS	5	0.5	1	25	2	-	30	60	30.67±7.02^{h}	19	Light yellow, loose, water stained
M7	1/2 MS	5	-	-	25	2	-	30	60	48.58±7.14^{e}	18	Yellow, granular, hard
M8	1/2 MS	5	-	-	25	2	-	30	60	52.53±6.5^{e}	18	Yellow, granular, hard
M9	1/2 MS	5	-	-	_	2	-	30	60	40.74±2.05^{g}	18	Yellow, grain, loose
M10	1/2 MS	5	-	-	25	-	-	30	60	51.22±1.58^{e}	18	Yellow particles
M11	1/2 MS	5	-	-	25	-	0.2	30	60	60.63±7.5^{d}	15	Light yellow, granular
M12	1/2 MS	5	-	-	25	-	0.4	30	60	67±3.61^{c}	13	Light yellow, granular
M13	1/2 MS	5	-	-	25	-	0.6	30	60	78.67±5.13^{a}	12	Yellow, grain, loose
M14	1/2 MS	5	-	-	25	-	0.8	30	60	73.08±2.56^{b}	12	Yellow, grain, loose

Note: Different lowercase letters in the same column in the table indicate significant differences at 0.05 level

1.4 Effects of different hormone combinations on adventitious buds induced by somatic embryos

When embryogenic callus were inoculated on differentiation medium containing different hormone combinations, it was shown that: When embryogenic callus were inoculated on differentiation medium containing different hormone combinations, it was shown that: Embryogenic callus could differentiate buds in different degrees on different hormone combination medium, and the somatic embryo differentiation ability on hormone combination medium containing 6-BA and NAA was significantly higher than that on other hormone combination medium (Table 4), and the bud differentiation ability decreased with the increase of 6-BA concentration. The results indicated that 0.2 mg/L 6-BA was the optimal concentration to induce embryogenic callus differentiation into buds. Table 4 shows the effect of hormone induction on papaya bud: 6-BA>Zeatin, NAA>IAA. The optimal medium for embryogenic callus differentiation of papaya was MBN 2:1 /2 MS+0.2 mg/L 6-BA+0.2 mg/L NAA+3% sucrose +3.6 g/L plant gel. The callus of papaya began to bud after 30 d culture on differentiation medium (Figure 3F; Figure 3G), which can grow to 3~4 cm after 3~4 months (Figure 3H; Figure 3I).

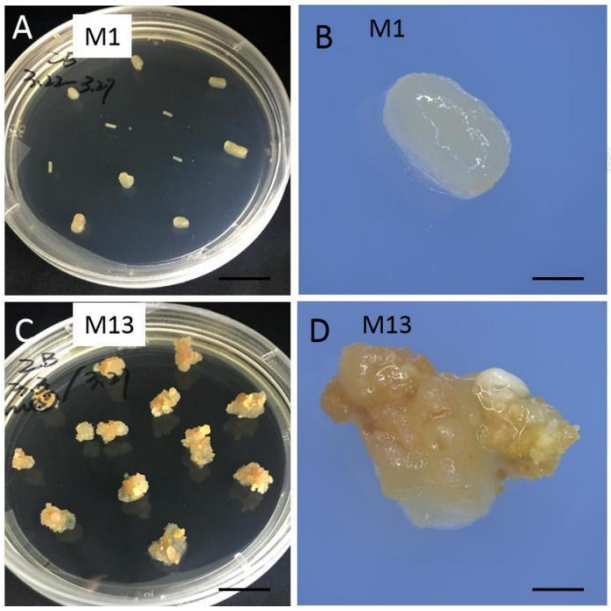

Figure 4 The state of hypocotyl explants on M1 and M13 induction medium

Note: A,B: Hypocotyl explants were cultured on M1 medium for 90 days; C,D: Hypocotyl explants were cultured on K5 medium for 30 days and then cultured on M13 medium for 60 days; A~C Scale=1 cm; B~D Scale=2 mm

Table 3 Embryogenic callus induction of different cultivar of papaya

Number	Cultivar	Rate of embryogenic induction (%)	Formation time of embryogenic callus (weeks)
1	Zhongbai	78.67±5.13^{b}	12
2	Hongfei	55.08±3.64^{e}	11
3	Hongling 1	59.47±1.5^{d}	11
4	Hongling 2	50.33±7.64^{f}	10
5	SunUp	73±1^{c}	11
6	Zhonghuang	85.23±7.55^{a}	10
7	AU9	75.33±2.52^{b}	12

Note: Different lowercase letters in the same column in the table indicate significant differences at 0.05 level

Table 4 Effects of plant growth regulators on the inducing callus differentiation

Medium number	Basic medium (mg/L)	A: 6-BA concentration 1/2MS (mg/L)	B: NAA concentration (mg/L)	C: Zeatin concentration (mg/L)	IAA concentration (mg/L)	Sucrose concentration (g/L)	Number of inoculated callus	Frequency of shoot regeneration (%)
MBN 1	MS	0.2	0.2	_	_	30	120	73.78±1.35^{b}
MBN 2	1/2 MS	0.2	0.2	_	_	30	120	84.31±3.24^{a}
MBN 3	1/2 MS	0.5	0.2	_	_	30	120	80.19±7.95^{a}
MBN 4	1/2 MS	1	0.2	_	_	30	120	76.55±2.23^{b}
MBN 5	1/2 MS	1	_	_	0.1	30	120	67±5.00^{c}
MBN 6	1/2 MS	0.5	_	_	0.1	30	120	69.33±4.04^{c}
MBN 7	1/2 MS	_	_	1	0.1	30	120	62.33±2.08^{c}
MBN 8	1/2 MS	0	0	2	0.1	30	120	65.71±4.45^{c}
MBN 9	1/2 MS	0	0	3	0.1	30	120	73.33±2.08^{b}

Note: Different lowercase letters in the same column in the table indicate significant differences at 0.05 level

1.5 Effect of IBA and rooting agent on rooting of rootless papaya seedlings

The rooting of tissue culture seedlings of papaya is very important for propagation. Inoculating sterile seedlings suitable for rooting on different rooting media (Table 5). The results showed that the rooting rate of papaya was low in different concentrations of IBA and NAA medium, and NAA did not promote the rooting of papaya. The rooting rate of papaya supplemented with IBA was significantly higher than that of IBA and NAA. The highest

rooting rate was 44% when IBA was 5 mg/L, and the rooting time was about 2 weeks, which was relatively short (Table 5). However, the high concentration of IBA made the leaves of the sterile seedlings turn yellow and fall off seriously, and the raw roots were puffed, and the viable plants could not be obtained in the later seedling training process. When IBA concentration was 1 mg/L, the root quality was good, but the rooting rate was only 36%, which was low (Table 5). The result indicated that 'Zhongbai' of papaya was difficult to root in culture medium. We attempted to directly induce rooting of sterile seedlings with root-growing powder. As can be seen from the results in Table 6, rooting rate of seedlings to be rooted after treatment with rooting solution of 100 mg/L was the highest, reaching 47% (Table 6). After transplanting to the nutrient soil for about 20 to 30 days, the roots will be successful and the seedlings will grow significantly (Figure 5). It can be seen from the experimental results that rooting in nutrient soil directly after treatment with rooting solution can significantly improve the rooting rate of 'Zhongbai' papaya, and shorten the rooting time, no longer need to practice seedlings, and can save about 2 months of time.

Figure 5 Tissue cultured seedlings grown at different times

Note: A: Tissue culture seedlings were grown in soil for 14 days; B: tissue culture seedlings were grown in soil for 28 days; C: tissue culture seedlings were grown in soil for 50 days; Scale = 1cm

Table 5 Effect of kinds and ratios of plant growth regulators on rooting of rootless seedlings in bottles

Medium number (mg/L)	Basic medium 1/2MS	IBA concentration (mg/L)	NAA concentration (mg/L)	Sucrose (g/L)	Number of inoculated seedings	Rooting rate (%)	Rooting time (week)	Growth status
R1	1/2 MS	0	0	30	48	0^{f}	0	Normal leaves
R2	1/2 MS	0.2	0.2	30	48	25.33 ± 3.06^{c}	4	Normal leaves
R3	1/2 MS	0.2	1	30	48	8.27 ± 2.60^{d}	4	Normal leaves
R4	1/2 MS	0.2	2	30	48	0^{f}	0	Normal leaves
R5	1/2 MS	0.2	3	30	48	4 ± 3.46^{e}	4	Yellow leaves
R6	1/2 MS	0.2	5	30	48	0^{f}	0	Yellow leaves
R7	1/2 MS	1	0	30	48	35.56 ± 2.92^{b}	3	Yellow leaves
R8	1/2 MS	2	0	30	48	8.32 ± 2.03^{d}	3	Yellow leaves
R9	1/2 MS	3	0	30	48	25.54 ± 1.58^{c}	2	Leaves turn yellow and fall off
R10	1/2 MS	4	0	30	48	25.18 ± 1.94^{c}	3	Leaves turn yellow and fall off
R11	1/2 MS	5	0	30	48	43.92 ± 4.48^{a}	2	Leaves turn yellow and fall off

Note: Different lowercase letters in the same column in the table indicate significant differences at 0.05 level

2 Discussion

There have been many reports on callus induction of papaya, mainly using different tissue parts of one variety as explants. But different varieties of papaya and different explants have different requirements for the combination of medium. In this study, 'Zhongbai', the main cultivar in Hainan, was used as the experimental material. Compared with other varieties, 'Zhongbai' had lower callus induction rate and slower callus growth rate. Therefore,

this study studied the effect of different media on the proliferation and differentiation induced by callus of 'Zhongbai', in order to provide an important research basis for the further genetic engineering improvement of 'Zhongbai' varieties.

Table 6 Effect of kinds and ratios of plant growth regulators on rooting in the soil

Medium	Rooting liquid (mg/L)	Number of inoculated seedlings	Rooting rate (%)
R13	50	200	53.15±1.11[a]
R14	100	200	47.12±2.72[b]
R15	150	200	35.67±2.52[c]
R16	200	200	32.81±2.97[c]

Note: Different lowercase letters in the same column in the table indicate significant differences at 0.05 level

2.1 The key factors of efficient induction of embryogenic callus of papaya

The explants of papaya are crucial to the induction of embryogenic callus. Cai et al. (2011) described that young embryos were the best materials for inducing embryogenic callus, but the induction efficiency of embryo stripping was low. In this experiment, the hypocotyl of seeds cultured directly under darkness was used as explants, which greatly increased the efficiency of explants preparation. According to the callus characteristics of papaya at different stages, the formation of embryogenic callus was divided into four stages: Explant expansion stage (Stage I), primary callus formation stage (Stage II), embryogenic callus formation stage (Stage III) and embryogenic callus propagation stage (Stage IV). The optimal combination of plant hormones and growth hormones is essential for the success of plant tissue culture. Different media were selected for different stages of callus induction. Akama et al. (1992) proved that explants bud on the medium with high cytokinin concentration, callus on the medium with nearly balanced auxin and cytokinin, and root on the medium with high auxin content. During the phase I-II of callus induction, explants were cultured on K5 medium containing hormone 2,4-D, KT, and the explants expanded, basic cell differentiation was established, and primary callus progenitor was formed (Motte et al., 2014). At the stage II-IV of embryogenic callus induction, the main inducing hormone in the medium was 2,4-D, and we proved that 2,4-D was the main hormone for callus induction in papaya. With the decrease of 2,4-D concentration, the callus induction rate decreased significantly, which was consistent with the previous report by Ding and He (2005).

The results of this study indicated that during callus induction stage I to II, KT in the medium was necessary for explants to expand and primary callus induction. At the same time, the induction of exogenous hormone KT directly affected the quality and number of primary callus formation. Studies have shown that simultaneous application of auxin and cytokinin can induce embryogenic callus formation (Sagare et al., 2000). In our study, it was demonstrated that cytokinin was required during callus induction in papaya, while KT was inhibitory during embryogenic callus formation and reproduction (Table 1; Table 2), which revealed the different hormone requirements of papaya callus at different developmental stages. During embryogenic callus induction, exogenous KT may inhibit the expression of some genes that promote somatic embryogenesis, and ultimately affect somatic embryogenesis. Our results also demonstrated that the two-step callus induction system was suitable for callus induction in 7 papaya varieties (Table 3), further indicating that the nature of different hormone requirements at different stages of callus induction in different papaya varieties is somewhat universal.

Armstrong G L et al confirmed that L-Pro significantly improved embryogenic callus rate (Fu et al., 2000). The embryogenic callus induction medium M11-M14 used in this experiment was significantly better than the medium without L-Pro in terms of the rate of healing and the quality of callus, indicating that the addition of L-Pro had a significant promoting effect on embryogenic callus induction. As a nitrogen source, L-Pro greatly accelerated the transformation of papaya callus into embryogenic callus. It is possible that the callus transformed L-Pro into other amino acids during this process, and at the same time enhanced the activity of phenylalanine ammonlyase and promoted the metabolism of phenylpropanes, which was conducive to the formation of embryogenic callus. During embryogenic callus proliferation of papaya, sucrose concentration in the medium not only acts as the main carbon source and energy substance, but also can regulate the osmotic pressure of the medium, thus affecting the

differentiation and growth of organs. In this study, adding high concentration of sucrose in embryogenic callus propagation medium played an important role in the rapid propagation of embryogenic callus.

2.2 The key technology to improve the rate of bud and rooting of somatic embryo differentiation of papaya

Li et al. (China Tropical Agriculture, (5): 58-60) also reported that 0.2 mg/L BA and 0.2 mg/L NAA were the most suitable hormone combinations for bud induction and differentiation of papaya. The optimal concentration of 'Zhongbai' bud differentiation was 0.2 mg/L BA and 0.2 mg/L NAA. These results indicated that the best bud induction hormone combinations were 6-BA and NAA, and the bud differentiation of different varieties of papaya could be achieved if the concentrations were both within the range of 0.1~0.3 mg/L. It also indicated that different varieties of papaya had similar hormone requirements during bud differentiation. When BA concentration is too high, foamy callus will appear at the stem base of the plant. Therefore, in the process of proliferation, the appropriate concentration of 6-BA to avoid the formation of callus at the stem base is very critical to obtain high quality successor plantlet of papaya.

When explants are cultured in callus induction medium for too long, the callus will not differentiate into buds, but take root directly. Therefore, minimizing the time of callus induction is a key step for callus regeneration (Che et al., 2007). In our experiment, two-step induction was used in callus induction stage, which significantly shortened the formation time of embryogenic callus of papaya, thus avoiding a long time of callus induction, and was more conducive to bud differentiation in the next stage.

Inducing rooting of tissue culture plantlet of papaya is a key technique in propagation and genetic transformation. Zhou et al. (2005) reported that KT, NAA and IBA rooting medium could induce high-quality roots, and the rooting rate reached 85%. However, Wang et al. (2009) showed that IBA was more effective than NAA in inducing the rooting of proliferating buds, and NAA was not conducive to the rooting of proliferating buds and the growth of transplanted seedlings of papaya. These results indicate that IBA is the most important growth regulator to promote rooting of papaya seedlings. However, tissue culture of papaya was genotype-dependent (Yuan et al., 2004), especially in the aspect of rooting difficulty and low survival rate of transplanting. During the rooting study on 'Zhongbai', we found that both NAA and IBA were used for rooting, and the rooting rate decreased with the increase of NAA concentration (Table 6). Compared with NAA, IBA was more effective in inducing the rooting of proliferative buds, but the rooting efficiency of IBA was lower (only 44%), and the root quality was poor.

In order to further explore the rooting induction conditions of 'medium white' papaya, we used rooting liquid to directly soak the 'medium white' tissue culture seedlings for 3 h, and then directly root in the nutrient soil. It was found that the rooting rate increased to 53%, and the rooting time of tissue culture bottle and seedling training was reduced. The root-growing powder had a great effect on the tissue culture of 'medium white' seedlings, especially on the cultivation and rapid rooting after transplanting. Commercial ABT root growth powder may induce adventitious root formation by enhancing and regulating the content and important activity of plant endogenous hormones. Therefore, it is very important to use commercial root powder to improve the efficiency of genetic transformation and reduce the cost of factory commercial production.

There are many mechanisms involved in embryogenic callus induction stage of papaya, but there are few reports on this. In the next experiment, we will analyze the gene expression of materials at different stages of embryogenic callus development by RNAseq sequencing to find the differentially expressed genes of explants during dedifferentiation and the key genes for embryogenic callus formation. These key genes will be of great significance to elucidate the molecular mechanism of somatic embryogenesis in papaya.

3 Materials and Methods

3.1 The materials of papaya

The papaya cultivar 'Zhongbai' was provided by South Subtropical Crops Research Institute, Chinese Academy of Tropical Agricultural Sciences. 'Hongfei', 'Hongling 1', 'Hongling 2' were provided by Guangzhou Fruit Tree Science Research. 'Zhonghuang', 'AU9' and 'SunUp' were germplasm resources preserved in the Center for Genomics and Biotechnology of Fujian Agriculture and Forestry University.

3.2 The establishment of aseptic lines and the acquisition of explants

150 seeds of 'Zhongbai' papaya were taken into a sterilized tissue culture bottle, disinfected with 75% alcohol for 1 min, washed with sterile water for 3~5 times, and then disinfected with 3.5% NaClO solution for 20 min, washed with sterile water for 3~5 times. Finally, it was transferred to 0.1% mercury litres for disinfection for 10 min and then washed with sterile water. After cleaning, the seeds were transferred to a sterile conical flask with 100 mL 20% NaClO solution and placed in a shaker at 180 r/min at 28℃ for 4 h. The above treated seeds were taken out and replaced with 1 mol/L KNO_3 solution and placed in a shaker at 28℃ and 180 r/min for 24 h. After this treatment was completed, the seeds were washed with sterile water 3-5 times, replaced with sterile water containing carbobenzylpenicillin and placed in a shaker at 30℃ at 120 r/min. The sterile water was changed every day until the seeds sprouted. When the seeds grew to about 0.5 cm, they were inoculated on 1/2 MS and cultured at 28℃ in dark chamber for 7~10 days. After that, young hypocotyls were taken as explants.

3.3 The acquisition of primary callus

The hypocotyl was cut into 8~10 mm segments and placed on 1/2 MS basal medium. Callus induction medium with different concentrations of 2, 4-D and KT was added to induce explants. Each treatment was inoculated with 6 dishes and each vial was inoculated with 10 explants for 3 replicates. The number of expanded explants was counted and photographed 10 days after inoculation. After 30 days of inoculation, the number of callus formed was counted and the results were analyzed. The optimal culture medium for callus induction was K5: 1/2 MS supplemented with 5 mg/L 2, 4-D, 0.5 mg/L KT, 30 g/L sucrose, 3.6 g/L plant gel, pH 6.0.

3.4 Acquisition and propagation of embryogenic callus

The explants were cultured on K5 medium for one month and then transferred to embryogenic callus induction medium with different substances, which mainly induced embryogenic callus. Each treatment was inoculated with 6 dishes and each vial was inoculated with 10 explants obtained from K5 medium for 3 replicates. The number of embryogenic callus growth was counted at 90 days after inoculation. It was transferred three times. The optimal culture medium for embryogenic callus was M13: 1/2 MS supplemented with 5 mg/L 2, 4-D, 0.6 mg/L L-Pro, 25 mg/L vitamin, 3% sucrose, 3.6 g/L plant gel, pH 6.0. The tender yellow and granular embryogenic callus growing on the callus surface on M13 were selected to Ci medium (1/2 MS+2, 4-D 10 mg/L+ sucrose 70 g/L) for propagation, and the callus without long bud and brown or white color were removed. The callus on Ci medium propagated once every 3 to 4 weeks. During the propagating process, the callus would be removed, such as browning, hardening and white sprouting tissue, leaving the callus with good growth to continue propagating.

3.5 Acquisition of embryogenic callus of different varieties of papaya

Six varieties, 'Hongling 1', 'Hongling 2', 'Hongfei', 'SunUp', 'Zhonghuang' and 'AU9', were prepared for explants according to the above methods, and then placed on K5 and M13 medium for culture according to the above methods, and the callus induction of each variety was calculated.

3.6 Induction and differentiation of embryogenic callus

The expanded light yellow callus were transferred to differentiation medium, which was 1/2 ms supplemented with different combinations of 6-BA, NAA, Zeatin and IAA. Each treatment was inoculated with 6 dishes and each vial was inoculated with 20 clusters of embryogenic callus for 3 times. After 30 days of inoculation, the optimal medium for differentiation was MBN 2:1 /2 MS supplemented with 0.2 mg/L 6-BA, 0.2 mg/L NAA, 3% sucrose, plant gel 3.6 g/L and pH 6.0. The callus was replaced with a new medium on the differentiation medium every four weeks or so. When the yellow buds grew into green buds, transferred the green buds to the culture bottle to grow. When the plants grew to 2 cm tall, the leaves became larger and the stems became thicker, they were ready to take root.

3.7 Rooting of tissue culture seedlings

The thriving tissue culture seedlings were transferred to 1/2 MS basal medium, and different concentrations of IBA and NAA were added to induce rooting. Each treatment was inoculated with 6 vials and 8 plants were inoculated in each vial with 3 replicates. Adding different concentrations of IBA, the rooting rate of 'Zhongbai'

was very low, and no optimal rooting medium was selected. The tissue culture seedlings were cultured to a height of 3-5 cm. Seedlings to root with different concentrations of strong rooting vacuoles were then transplanted into nutrient soil, covered and moisturized. It takes about 20 days to take root. Through rooting rate analysis, it was found that 100 mg/L rooting solution had the best rooting effect.

3.8 Processing of data

Excel software was used to calculate the mean value and standard deviation, and SPSS 22.0 was used for difference significance analysis.

Authors' contributions

ZXB was the executor of the experimental design and experimental research of this study. ZXB, ZQX, YLY, FHM and WYW completed the data analysis and the writing of the first draft of the paper. ZLP participated in experimental design and analysis of experimental results. YJJ was the proposer and leader of the project, directing experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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