Mass-production of Papaya (*Carica papaya* L.) Saplings Using Shoot-tip Culture for Commercial Use

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Abstract

Culture conditions for shoot tips, excised from mature papaya trees, were investigated to develop shoot tip culture system. The aim was to proliferate saplings of newly developed cultivars for commercial use in association with a breeding program. Shoot tips of 'Wonder Blight' were grown on initiation medium consisting of MS (1962) basal components with 0.1M sucrose. Addition of 5 - 20 μM 6BA and NAA was effective for multiplication of the shoots. Afterwards, the shoots were transferred to a medium with 2-ip for further development. Addition of 10 - 20 μM 2-ip was effective for shoot development. Developed shoots from subculture were further examined for rooting with high (10mM) and low (10 μM) concentrations of IBA for 5 sec and 10 days treatments, respectively. The shoots were then put into 'Oasis (LC-1-288: 5554)' growing medium filled with tap water for rooting. The treatment with 10 μM IBA for 10 days showed higher rooting rates, though the 10mM IBA for 5sec treatment was cost effective. Rooting was also affected by size of the shoots; well developed ones showed higher rooting and faster recovery than weak ones. More than 85% of the plantlets developed roots and 80% of those recovered after acclimatization.
Keywords: callus, *in vitro* proliferation, multiple shoots, shoot growth, tissue culture

Introduction

About thirty typhoons appear annually in Japan. Many of them hit Ryukyu Islands severely, where agriculture is one of the main industries. Because of the prevailing warm temperature, tropical and subtropical fruits such as pineapple, banana, and papaya have been grown for centuries in the area. To protect crops from typhoon dam-

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ages, fruit trees such as mango (*Mangifera indica* L.) and papaya (*Carica papaya* L.) are grown in strong greenhouses in Ryukyu Islands. The greenhouses are built much stronger than those in other parts of Japan, including those found in southern Kyushu, the southern-most main island of Japan.

Papaya is traditionally considered as vegetable as in Southeast Asia and immature green papaya fruits are sold in local markets for cooking. However, dessert papaya is also becoming very popular among Japanese consumers. Papaya plants are usually grown in greenhouses or covered with screen to protect them from typhoons in order to produce high-quality fruits. In such systems, papaya plants are planted in an inclining manner otherwise they would reach the roof within two years of planting. This system, however, cannot be continued for more than three or four years, because the trunk cannot stand the heavy weight of the top of the trees. Grafting is adapted to reduce the height of plants as well as to proliferate androgynous and female plants. However, it is rather difficult to achieve the objectives with the existing cultivars. Superior and dwarf cultivars are needed for both vegetable and fresh fruit markets. Tissue culture have the possibility for overcoming problems of lack of efficient preparing materials associated with breeding programs for cultivar improvement.

The present paper describes the tissue culture system to optimize proliferation protocols for papaya saplings for commercial use.

Materials and Methods

Plant Materials

One-year-old 'Wonder Blight' papaya cultivar grown in a greenhouse was used in the experiment. Twenty plants were cut back 50cm from the top and newly developed shoot sprouts of 2 to 3cm long were collected. Apical shoots were mainly used, though the axillary shoots were also collected for the experiments on sterilization of the materials. The materials were used randomly and about 50 - 100 samples were used for each treatment.

Sterilization of the Materials

Three treatments were compared, which are as follows;

1. Sprouting shoots were sprayed with 70 % (v/v) ethanol well before sampling. They were cut with a knife sterilized with 70 % ethanol and taken into the laboratory.
2. Sprouting shoots were sprayed with 0.05% streptomycin sulfate once a day for 5 days before sampling. They were cut with a knife sterilized with 70 % ethanol and taken into the laboratory.
3. Shoots were cut with a knife sterilized with 70% ethanol and taken into the laboratory to use as the control.

After taking into the laboratory, shoots in all the three treatments were immersed 5 seconds in 70% ethanol for surface sterilization. Then, they were treated with 0.5%

sodium hypochlorite solution for 10min. After rinsing three times with sterilized water, the tips were trimmed into small pieces which included the meristem. The tips were excised to make about 3mm long and immediately placed on the surface of 7ml of medium in 20 x 100mm culture tubes.

Culture Conditions for Shoot Proliferation and Growth

The MS (MURASHIGE and SKOOG 1962) basal medium with 0.1M sucrose was used otherwise stated. Before autoclaving, the media were adjusted to pH5.7 and solidified with 0.9% agar. Various plant growth regulators were also added to the media as mentioned below. Cultures were incubated at 25 ± 1° C under a 16-hour photoperiod using warm white fluorescent lamps (25 - 35μE・m^{-2}・s^{-1}). They were subcultured at about 3- to 4-week intervals. Extent of the shoot proliferation was estimated with the number of shoots formed, while shoot growth was evaluated by the length of the shoots formed.

1. NAA and 6BA

Effects of α-naphthaleneacetic acid (NAA) and 6-benzylaminopurin (6BA) on shoot proliferation and growth were examined with 0.1 - 20μM combinations as indicated in Fig. 1 and 2.

High concentrations of both NAA and 6BA combinations were also examined for callus and shoot formation. Two media, containing 50μM of each of NAA and 6BA, and 20μM of both were examined as the first medium for stimulation of callus and shoot formation with shoots subcultured on a growing medium containing 10μM 2iP. After 3weeks, they were transferred onto the second media containing 1 - 20μM of NAA and 6BA as in Fig. 4.

2. Sucrose

The basal medium containing 5μM NAA and 6BA was used for the experiment. Various concentrations of sucrose (0.02 - 0.2M) were examined on callus formation and shoot proliferation as mentioned in Fig. 5 and 6.

3. GA_3

The basal medium containing 5μM NAA and 6BA were used for the experiment. Gibberellic acid (GA_3) of 5 - 50μM concentrations was added to the medium and examined on shoot proliferation and growth as in Fig. 7 and 8.

4. 2iP

The medium containing different concentrations, 0.1 - 50μM 6-(γ, γ-dimethylallylamino) purine (2iP), was examined on shoot growth as in Fig. 9.

Root Induction

Shoots measuring more than 1cm long on a medium with 10μM 2iP were used for the experiments with Indole-3-butyric acid (IBA). A treatment with high concentration of IBA for short time (HS) and that with low concentration of IBA for long time (LL) were examined on root induction from the shoots. For HS, basal cut ends of

shoots were immersed 5 seconds in 50% ethanol containing 10mM IBA. For LL, shoots were cultured with MS medium containing 10μM IBA for 10 days. After each treatment, shoots were inserted into 'Oasis Growing Medium (LC-1-288: 5554)' filled with tap water.

Results and Discussion

Sterilization of the Materials

Spraying with 70% ethanol before sampling was the most effective method of sterilization for both apical and axillary shoots followed by that with 0.05% streptomycin sulfate for 5 days (Table 1). Control treatment had the highest contamination rates compared to the other two methods. Noticeably, apical shoots showed considerably higher survival rates than that of axillary shoots in all the treatments.

Table 1. Effects of sterilization methods on contamination of cultures.

Treatments	Apical shoots		Axillary shoots	
	No. of shoots cultured	No. of shoots uninfected (%)	No. of shoots cultured	No. of shoots uninfected(%)
Ethanol[1]	94	67 (71.3)	36	8 (22.2)
Streptomycin sulfate[2]	91	41 (45.1)	32	5 (15.6)
Control[3]	88	29 (33.0)	37	4 (10.8)

1 : Spraying with 70% (v/v) ethanol just before sampling.
2 : Spraying with 0.05% (w/v) streptomycin sulfate once a day for 5days before sampling.
3 : No treatment before sampling.

Contamination is the most critical problem in tissue culture when using field materials. LITZ and CONOVER (1978) obtained less than 5% uncontaminated cultures from field papaya plants. MONDAL *et al*. (1990) were able to establish more than 70% uninfected cultures from fruit bearing papaya plants in the field with 750mg・L^{-1} gentamycin solution by spraying once a day for 7days. However, in our experiment, spraying of 0.05% streptomycin sulfate for 5 days was not so effective as we recorded 45.1% uninfected cultures for apical shoots and only 15.6% for axillary shoots. Contrary, spraying 70% ethanol to the apical shoots before sampling was the most effective method for sterilization of field papaya plants. Probably, excessive usage of antibiotics can be reduced by adopting this inexpensive method.

Culture Conditions for Shoot Proliferation and Growth

1. NAA and 6BA

No clear results among the treatments were obtained after two times of subculture for 30 days on shoot proliferation (Fig. 1). Shoots on the media containing relatively lower concentration of NAA and 6BA showed vigorous growth (Fig. 2). However, shoots subcultured on the media with higher concentrations, such as 20μM

NAA and 6BA, showed chlorosis after 30 days and the shoot growth became poor (data not shown). Shoots subcultured on a media containing 5μM NAA and 6BA showed good proliferation after several subcultures, though the shoots did not show individual growth (Fig. 3). The results indicated that the media with NAA and 6BA might be effective for shoot proliferation, but not for shoot growth. LITZ and CONOVER (1978) observed the greatest number of papaya plantlets with a medium containing 2μM 6BA and 1μM NAA. They observed plantlets from the shoots after transferring directly to root induction medium. The size of shoots, however, should be bigger for commercial use, as mentioned below.

Shoots transferred onto the medium containing 50μM NAA and 6BA turned into whitish-yellow and their survival rates were between 60 to 75% after subculture (Fig. 4). However, all the shoots on the medium with 20μM NAA and 6BA survived after

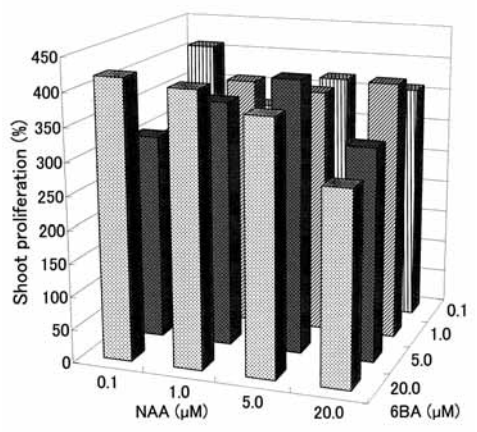

Fig. 1. Effects of NAA and 6BA on shoot proliferation after 2 times of 30 day culture.

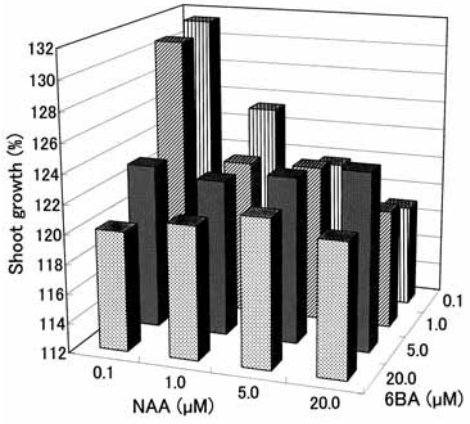

Fig. 2. Effects of NAA and 6BA on shoot growth after 2 times of 30 day culture.

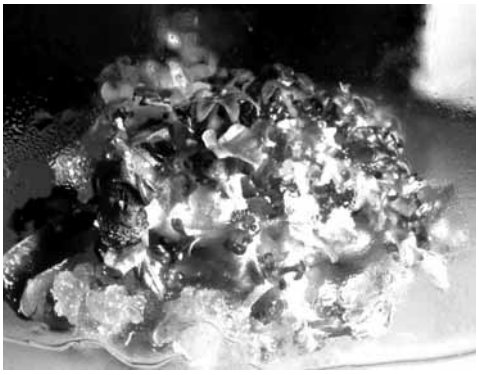

Fig. 3. Shoot proliferation on the medium with 5 μM NAA and 6BA.

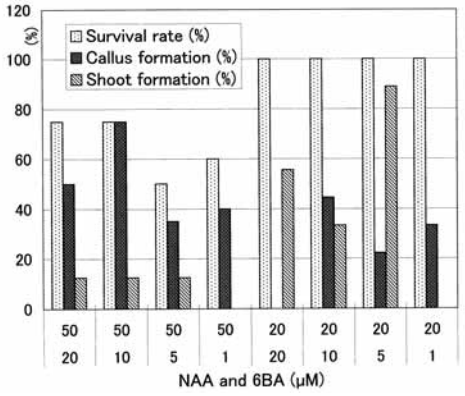

Fig. 4. Effects of high concentration of NAA and 6BA on survival rate, and formation of callus and shoots.
The upper row of x-axis is the first medium and the lower is the second medium. Same concentration of both NAA and 6BA was added.

subculture. Furthermore, many new shoots were formed from various parts of the shoots transferred from the medium containing 20μM NAA and 6BA. This might suggest that the medium containing high concentration of NAA and 6BA, such as 50μM, should not be used as the first medium, whereas lower concentration such as 20μM of NAA and 6BA might be effective to stimulate shoot proliferation.

2. Sucrose

Higher concentration of sucrose was effective for callus formation (Fig. 5) and shoot proliferation (Fig. 6). However, shoots proliferated on the medium with 0.2M sucrose were vitrified. Addition of 0.05 - 0.1M sucrose to the medium was effective for callus and shoot proliferation in this experiment. KATAOKA and INOUE (1992) observed that addition of 1 - 3 % sucrose did not affect shoot growth in papaya. Sucrose might play more important role for shoot proliferation through callus formation than for shoot growth in papaya. Higher concentrations of sucrose stimulated callus formation in citrus anthers (HIDAKA 1984) and various saccharides affected embryogenesis from citrus callus (HIDAKA and OMURA 1989). Various concentrations of sucrose as well as other saccharides should be examined on papaya tissue cultures.

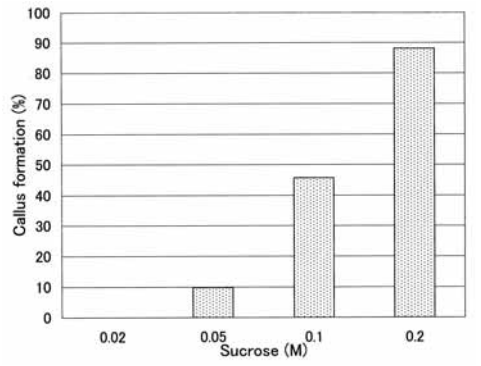

Fig. 5. Effects of sucrose on callus formation from shoots.

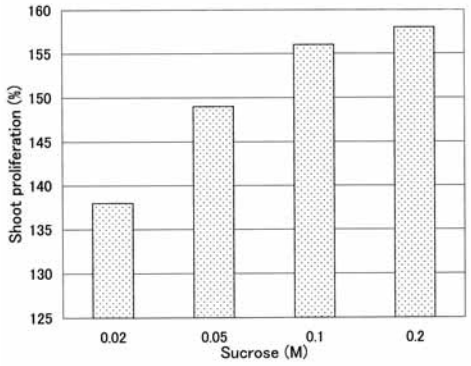

Fig. 6. Effects of sucrose on shoot proliferation.

3. GA_3

Addition of GA_3 suppressed shoot proliferation (Fig. 7), and 10μM GA_3 was relatively effective for shoot growth, though the difference among treatments were not so significant (Fig. 8). WINNAAR (1988) used GA_3 to stimulate growth of compact plantlets. However, the shoots treated with GA_3 looked weaker. Other plant growth regulators or factors could be tested to stimulate shoot growth.

4. 2iP

Shoots transferred onto the medium with 10μM 2iP showed vigorous growth (Fig. 9 and 10). Our results corroborate with that of KATAOKA and INOUE (1992), who also observed vigorous growth with 0.5mg/L 2iP. Addition of GA_3 might not be needed if shoots are transferred on to the medium with 10μM 2iP.

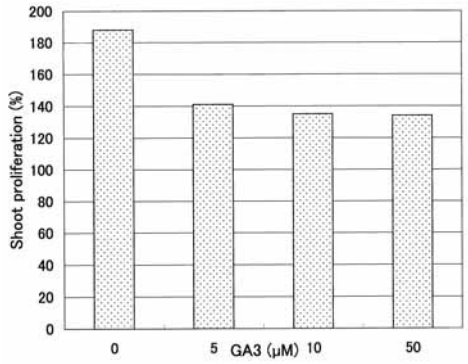

Fig. 7. Effects of GA_3 on shoot proliferation.

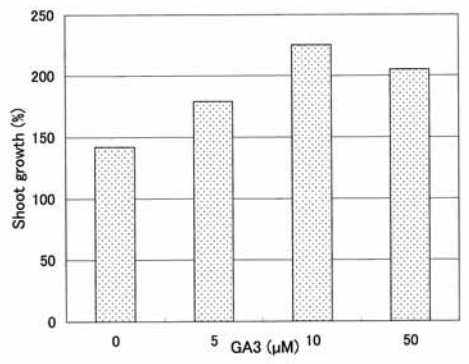

Fig. 8. Effects of GA_3 on shoot growth.

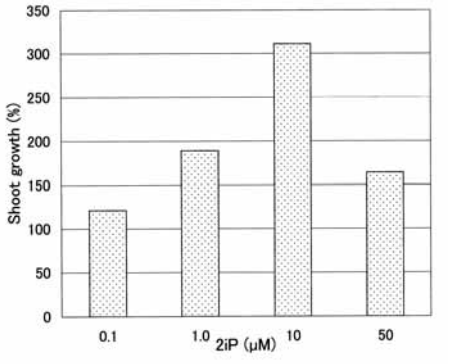

Fig. 9. Effects of 2iP on shoot growth.

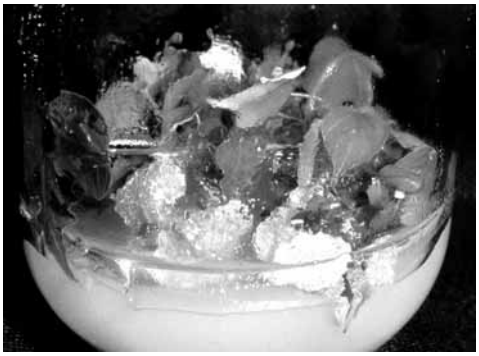

Fig. 10. Shoots growing on a medium with 10 μM 2iP.

Root Induction

Higher rooting rates of the shoots were observed on a medium containing 10μM IBA than that immersed in 10mM IBA solution (Table 2). YU *et al*. (2000) also observed efficient root induction with agar medium containing low-concentration of IBA, however, immersing method might be cost effective in case of commercial use because it takes only five seconds and do not need cultures *in vitro*. Shoots more than

Table 2. Effects of treatments and size of shoots on root induction and survival rate in 3 weeks after potting.

Treatments	No. of shoots cultured	No. of shoots rooted	No. of shoots potted	Survived after potting[3]
High concentration with short time[1]	54 (26)	42 (23) 77.7 (88.5)	40 (23) 74.1 (88.5)	37(21)
Low concentration with long time[2]	50 (24)	47 (24) 94 (100.0)	45 (22) 90 (91.7)	41 (20) 82 (83.3)

Lower number of each column: No. of shoots treated/No. of shoots cultured x 100
Numbers parenthesized were of shoots more than 2cm long.
1: Basal cut ends of shoots were immersed 5 seconds in 50% ethanol containing 10mM IBA.
2: Shoots were cultured on a medium containing 10μM IBA for 10days.
3: Final surviving was determined after 3weeks of potting.

2cm long showed higher rooting and survived rates than the smaller ones. The results suggest that the size and vigor of each shoot affect rooting of papaya shoots culture. DREW and MILLER (1989) also observed that good rooting occurred in shoots growing actively. Furthermore, inserting such vigorous shoots after IBA treatment into 'Oasis Growing Medium' was very effective for rooting especially for commercial use when potting (Fig. 11).

The methods described in this paper are being applied to the proliferation for new cultivars (Fig. 12). Despite low quality of imported fruits, arising form premature harvesting for export, large amount of tropical and subtropical fresh fruit is importing into Japan and the trend is increasing. Since Japanese consumers prefer safe and high quality fruit, and thus has strong demand for exotic fruits, the papaya industry in Japan is supposed to be expanded in the near future.

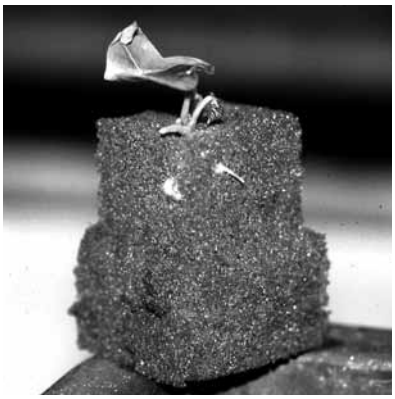

Fig. 11. Rooting in 'Oasis Medium'.

Fig. 12. Papaya plants proliferated with tissue culture in a farmer's greenhouse.

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